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Vectors Specifically to Prostate Cancer Cells

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## Introduction

This grant proposal was focused upon the use of retroviral receptor-ligand bridge proteins to target therapeutic retroviral vectors specifically to markers that are expressed on the surfaces of prostate cancer cells. These projects were aimed at targeting retroviral vectors via heregulin receptors which are overexpressed on prostate cancer cells and via TAG-72, a prostate cancer cell-specific marker. In addition, we proposed to improve the efficiency of targeted viral infection by employing bridge proteins that contain function-enhancing amino acid substitutions in the retroviral receptor domain and proposed generating retroviral vectors containing novel suicide genes for therapeutic purposes.

## Body

There were four specific aims proposed under this grant award.

**Specific Aim 1. To determine whether a soluble protein composed of the extracellular domain of TVA fused to the EGF-like region of neuregulin (heregulin) $2\beta$  can target retroviral infection when attached to the ErbB3 receptor on the surfaces of prostate cancer cells.**

As proposed in the original grant application, a gene encoding a TVA-heregulin protein (TVA-her $\beta$ 1) was constructed. For convenience purposes, heregulin $\beta$ 1 was chosen as the ligand for this construction, as opposed to heregulin- $2\beta$ . This alteration to the construct did not in any way affect the specificity of this protein, since both of these ligands bind to the same cellular receptors. A manuscript describing this work was recently accepted for publication (Appendix #4).

TVA-her $\beta$ 1 was produced and it bound specifically to the surfaces of transfected NIH 3T3 cells (N234 cells) that express heregulin receptors, but did not bind to 3T3 cells that lack these receptors (Fig. 1, Appendix #4). In addition, TVA-her $\beta$ 1 binding to N234 cells was competed in the presence of another retroviral receptor-ligand bridge protein (TVBs3-her $\beta$ 1) containing the same ligand domain (Fig. 3A, Appendix #4). By contrast, binding of TVA-her $\beta$ 1 to N234 cells was not affected by the presence of another retroviral receptor-ligand bridge protein (TVBs3-VEGF) that had a different ligand domain, namely that of vascular endothelial growth factor (VEGF) (Fig. 3A, Appendix #4). These results confirmed that the TVA-her $\beta$ 1 bridge protein attaches specifically to N234 cells via cell surface heregulin receptors.

The TVA-her $\beta$ 1 protein was tested for its ability to mediate the entry of subgroup A-ALV retroviral vectors encoding the enhanced green fluorescent protein (EGFP) specifically into N234 cells. For control purposes, these experiments were also performed with control NIH 3T3 cells that lack heregulin receptors as well as with transfected NIH 3T3 cells (NIH3T3-TVA cells) that expressed a transmembrane form of the TVA receptor, thus serving as an important control for 100% infection efficiency (Table 1, Appendix #4). Infection was monitored by flow cytometry to detect the EGFP-expressing cells.

These studies revealed that the TVA-her $\beta$ 1 protein mediated selective virus entry into N234 cells that express heregulin receptors. Approximately 46-fold higher levels of infection were achieved with N234 cells that had been incubated with the bridge protein as compared with untreated N234 cells (Table 1, Appendix #4). Furthermore, the level of infection that was achieved was quite efficient, being only 20-fold down from the maximum level that would be expected based upon control studies performed with NIH3T3-TVA cells (Table 1, Appendix #4). By contrast to N234 cells, NIH 3T3 cells displayed only background levels of infection either in the absence or presence of the bridge protein (Table 1, Appendix #4), confirming that the bridge protein mediates infection via cell surface heregulin receptors.

These studies have clearly shown that it is possible to achieve highly selective and efficient infection of cells via cell surface heregulin receptors using a TVA-her $\beta$ 1 bridge protein. There is now every reason to expect that this system can be used to deliver retroviral vectors into prostate cancer cells that overexpress heregulin receptors.

**Specific Aim 2. To determine whether a soluble protein composed of the extracellular domain of TVA fused to a single chain antibody that binds specifically to the TAG-72 protein can target retroviral infection to cells that express this surface marker.**

The objective of these studies was to test the feasibility of retroviral targeting via a retroviral receptor-single chain antibody bridge protein. In the original grant proposal we proposed testing such a protein with a single chain antibody-domain targeted against TAG-72, a prostate cancer cell surface marker. However, we subsequently reasoned that the MR1 single chain antibody that is directed against EGFRvIII, a mutant form of the human epidermal growth factor receptor would be much more useful for this purpose. EGFRvIII is a tumor-specific marker that is formed as a result of a gene deletion/rearrangement that occurs during tumor biogenesis. EGFRvIII is expressed on a variety of different cancer cell types including prostate cancer, glioblastoma, ovarian cancers and breast cancers. Thus, EGFRvIII is a preferred target for the development of retroviral targeting systems that are directed toward a number of different cancers including those of the prostate.

The following description of the study summarizes the major conclusions of a published study (Snitkovsky 2000 J. Virol. 74: 9540-9545) (Appendix #2). The TVA-MR1 protein was generated and was shown to bind specifically to the EGFRvIII protein expressed on cell surfaces (Figs. 1 and 2, Appendix #2). Furthermore, this protein specifically mediated the infection of EGFRvIII-expressing cells by subgroup A-ALV retroviral vectors (Table 1 and Figs. 3 and 4, Appendix #2). The level of targeted infection that was achieved was quite high, representing 8.5% of that level obtained with positive control cells that expressed the transmembrane form of TVA (Table 1, Appendix #2). Therefore, we conclude that ALV-A vectors can be selectively targeted toward cells that express EGFRvIII using the TVA-MR1 bridge protein.

**Specific Aim 3. To determine whether increased levels of retroviral vector infection via ErbB3 and/or TAG-72 can be achieved by introducing into the soluble TVA fusion proteins, amino acid substitutions that increase the activity of the wild-type retroviral receptor.**

We showed that introducing two mutations that were described in the original grant proposal (W48F and W48Y) into the TVA domain of the TVA-EGF bridge protein (Snitkovsky 1998 Proc. Natl. Acad. Sci. USA 95: 7063-7068) led to a 2- to 3-fold enhancement of viral entry into cells that expressed the EGF receptor. Given this modest effect, we decided not to pursue introducing these mutations into other TVA-ligand bridge proteins. Instead, we decided to test the activity of a TVA-vascular endothelial growth factor (VEGF) bridge protein. VEGF is an attractive ligand for this purpose because its receptors are expressed in endothelial cells of the tumor vasculature and thus it may become an important reagent for delivering retroviral vectors into cells with the aim of cutting off the blood supply to tumors. This work has been published (Snitkovsky 2001 J. Virol. 75: 1571-1575) (Appendix #3).

The TVA-VEGF protein was produced and it bound specifically to cells that express a VEGF receptor, VEGFR-2 (Fig. 2, Appendix #3). Furthermore, the TVA-VEGF protein mediated specific and efficient infection by ALV-A vectors into cells that express VEGFR-2 (8.72 % of the level obtained with positive control cells that express a transmembrane form of TVA) (Appendix #3). We conclude that it is possible to target retroviral vectors specifically to cells that express VEGF receptors.

**Specific Aim 4. To construct retroviral vectors containing suicide genes and to assess the efficiency of cell-killing mediated by these vectors following infection via ErbB3 and/or TAG-72.**

While these experiments were important, we faced a more pressing issue with the retroviral receptor-ligand bridge proteins. Specifically, the aforementioned experiments were all performed *in vitro* with cells that had been preloaded with the bridge proteins before viral challenge. However, for this to become a feasible approach for an *in vivo* application, then targeted infection mediated by bridge protein-loaded virions would need to be demonstrated. We therefore set out to test this idea using retroviral vectors that were preloaded with a TVB-EGF bridge protein. This work has been published (Boerger 1999 Proc. Natl. Acad. Sci. USA 96: 9867-9872) (Appendix #1). Indeed, the TVB-EGF-loaded virions could infect cells that express EGF receptors specifically and with a high efficiency (the level of targeted infection that was achieved in cells that express a kinase-deficient form of the EGF receptor was higher than that obtained with control cells that expressed a transmembrane form of the TVB receptor (Table 2, Appendix #1). Furthermore, the TVB-EGF loaded virions could be produced directly from viral packaging cells, retaining their cell-type specificity (Fig. 2, Appendix #1). We conclude from these studies that it is possible to generate retroviral receptor-ligand bridge protein-loaded retroviral vectors and have these vectors delivered to cells that express the cognate ligand receptor.

**Research Accomplishments**

Targeted retroviral infection has been achieved via:

- heregulin receptors through the use of a TVA-heregulin fusion protein
- EGFRvIII, a tumor specific-form of the EGF receptor, using a TVA-single chain antibody fusion protein
- VEGFR-2, a receptor that is expressed on tumor vasculature, using a TVA-VEGF fusion protein
- TVA-EGF-loaded retroviral vectors, demonstrating that virions preloaded with a retroviral receptor-ligand bridge protein can be targeted to specific cell types.

**Reportable Outcomes**

**Publications**

Appendix #1. Boerger, A.B., Snitkovsky, S., and Young, J.A.T. Retroviral Vectors Preloaded with a Viral Receptor-Ligand Bridge Protein Are Targeted to Specific Cell Types. Proc. Natl. Acad. Sci. U.S.A., 96: 9867-9872, 1999.

Appendix #2. Snitkovsky, S., Niederman, T.M.J., Carter, B.S., Mulligan, R.C., and Young, J.A.T. A TVA-Single Chain Antibody Fusion Protein Mediates Specific Targeting of an ALV Vector to Cells Expressing a Tumor-Specific Form of the EGF Receptor. J. Virol., 74: 9540-9545, 2000.

Appendix #3. Snitkovsky, S., Niederman, T.M.J., Mulligan, R.C., and Young, J.A.T. Targeting ALV-A Vectors using a TVA-VEGF Bridge Protein. J. Virol., 75: 1571-1575, 2000.

Appendix #4. Snitkovsky, S., and Young, J.A.T. Targeting Retroviral Vector Infection to Cells That Express Heregulin Receptors using a TVA-Heregulin Fusion Protein. *Virology*, in press, 2001.

Appendix #5. Young, J.A.T. Targeting Retrovirus Vectors using Molecular Bridges. Chapter in *Vector Targeting for Therapeutic Gene Delivery*, D. Curiel and J.T. Douglas, editors, Wiley Press.

### **Meeting Abstracts and Presentations**

Appendix #4. S. Snitkovsky, A. Boerger, J.A.T. Young. Targeting Infection by Retroviral Vectors using Retroviral Receptor-containing Bridge Proteins. Gene Therapy Meeting, Cold Spring Harbor Laboratory, September 25-29, 2000.

### **Personnel Receiving Pay**

John A.T. Young, Sophie Snitkovsky, Adrienne Boerger

### **Graduate Degrees**

**Sophie Snitkovsky, Ph.D. in Virology.** Targeting of Retroviral Vectors to Specific Cell Types Mediated by Soluble Retroviral Receptor-Ligand Bridge Proteins. Harvard University, December 1999.

**Adrienne Boerger, Ph.D. in Microbiology and Molecular Genetics.** The Use of Retroviral Vectors Preloaded with a Retroviral Receptor-Ligand Bridge Protein for Cell Type-specific Viral Targeting and as a System for Studying Avian Leukosis Virus Entry. Harvard University, May 2000.

### **Conclusions**

The studies that have been supported by this grant have led to the generation of an efficient, specific and versatile system for targeting retroviral infection to specific cell types. Viral targeting has been achieved using retroviral receptor-ligand, and -single chain antibody bridge proteins that are directed toward various markers expressed on cancer cells, including those of prostate cancer. This work is summarized in a book chapter that I recently authored (Appendix #5). This work represents an exciting step forward for efforts aimed at targeting retroviral infection to cancer cells. However, there are still major hurdles that lie ahead before this system can become useful for delivering retroviral vectors to cancer cells *in vivo*. The most important obstacle to be overcome will be to generate sufficient amounts of bridge protein-loaded virions to target a substantial number of the cancer cells in the body. Nevertheless, the work that was supported by this award represents a critical first step toward this future application.

### **References**

References pertinent to this report are listed under "Reportable Outcomes".

### **Appendices**

Appendices 1-5 follow.

## Retroviral vectors preloaded with a viral receptor-ligand bridge protein are targeted to specific cell types

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Edited by Inder M. Verma, The Salk Institute for Biological Studies, San Diego, CA, and approved June 21, 1999 (received for review February 12, 1999)

**ABSTRACT** Successful targeting methods represent a major hurdle to the use of retroviral vectors in cell-specific gene-delivery applications. We recently described an approach for retroviral targeting with a retroviral receptor-ligand bridge protein that was bound to the cognate cell-surface ligand receptors before viral challenge. We now report a significant improvement made to this viral targeting method by using a related bridge protein, designated TVB-EGF, comprised of the extracellular domain of the TVB receptor for subgroup B avian leukosis virus fused to epidermal growth factor (EGF). The most important activity of TVB-EGF was that it allowed specific viral entry when preloaded onto virions. Furthermore, virions preloaded with TVB-EGF were thermostable and could be produced directly from virus-packaging cells. These data suggest an approach for targeting retroviral vectors to specific cell types by using virions preloaded with a retroviral receptor-ligand bridge protein and indicate that these types of bridge proteins may be useful reagents for studying the normal mechanism of retroviral entry.

One of the major challenges facing retrovirus-based gene-delivery systems is in the development of approaches for efficiently targeting viral infection to only relevant cell types. A number of approaches have been tested in an effort to overcome this problem, including chemical modification of viral envelope (Env) proteins (1), the use of antibodies to bridge viral Env proteins with specific cell-surface molecules (2, 3), and the use of recombinant Env proteins containing cell-specific ligands or single-chain antibodies (4–17). Although these approaches have allowed for some degree of cell-type-specific viral entry, the level of infection observed is usually too low to be considered useful for most retrovirus-based gene-delivery applications (18, 19). Thus, there is clearly a need to develop new and improved methods for retroviral targeting.

We recently reported an approach for retroviral targeting with a retroviral receptor-ligand bridge protein (20). The bridge protein tested was comprised of the low density lipoprotein receptor-related extracellular domain of the TVA receptor for subgroup A avian leukosis virus (ALV-A) fused to human epidermal growth factor (EGF). When this bridge protein was added to the surfaces of cells that express EGF receptors (EGFR), these cells became highly susceptible to infection by ALV-A vectors (20). EGF was chosen as the prototype ligand to test this viral targeting approach, because its interaction with EGFR has been extensively characterized biochemically. Furthermore, EGFR is a relevant target for this method of retroviral vector delivery, because this receptor and other related receptors such as c-erbB-2 and c-erbB-3 are often overexpressed or mutated in solid human tumors (21).

Here, we show the functional activities of another type of bridge protein comprised of the tumor necrosis factor receptor-related extracellular domain of the TVB receptor for ALV-B fused to EGF. Most importantly, we show that it is possible to target retroviral vectors specifically to cells that express EGFR by preloading virions with the TVB-EGF bridge protein.

### MATERIALS AND METHODS

**Plasmids.** A DNA fragment encoding the extracellular domain of TVBS3 (amino acid residues 1–155) was amplified by PCR from pBK7.6-2 template DNA (22) by using primers OAB2 (5'-CATTGTTCTCGAGATGCGCTCAGCTGCGC-TCCG-3') and OAB5 (5'-CATTGTTCCGCCGTGAGTGG-AGGAGCTGGAGGAG-3'). The PCR product was subcloned into the pCI expression vector (Promega) and ligated with a DNA fragment from plasmid pSS1 (20), encoding a glycine- and proline-rich linker region and human EGF. The resulting plasmid, pAB1, encodes TVB-EGF, and the sequence was validated by DNA sequencing (Department of Microbiology and Molecular Genetics DNA Sequencing Core Facility, Harvard Medical School).

A 2,208-bp *KpnI-BamHI* DNA fragment encoding a correctly spliced envA mRNA transcript (K. Ziegler, L. Connolly, and J.A.T.Y., unpublished work) was subcloned into a modified version of the pCI expression plasmid to generate pAB6. Plasmid pAB7 encoding ALV-B Env was then generated by replacing the *XhoI-ApaI* env fragment of pAB6 with the corresponding 925-bp *XhoI-ApaI* fragment of the subgroup B RAV-2 env. Plasmid pMMP-nls lacZ, containing a murine leukemia virus (MLV) vector encoding  $\beta$ -galactosidase (lacZ); plasmid pMD.old.gag.pol, encoding the MLV proteins Gag and Pol (23); and plasmid pCMMP.GFP/neo, containing the gene encoding green fluorescent protein (GFP), were generous gifts from J.-S. Lee and R. C. Mulligan (Children's Hospital, Boston).

**Cell Lines.** Human 293 cells were obtained from the American Type Culture Collection. B82 cells are a clonal line of mouse L cells, which lack EGFR. As described in refs. 20, 24, and 25, these cells were transfected with plasmids encoding either a kinase-deficient EGFR or wild-type EGFR to generate clonal derivatives designated M5 and T23 cells, respectively. The M5:S3/T cell line was obtained after stable transfection of M5 cells with plasmids encoding FLAG-epitope tagged TVBS3 and TVBT, formerly SEAR, a subgroup E-specific ALV receptor (ref. 26 and H. Adkins and J.A.T.Y., unpublished work). Expression of TVBS3 in this cell line was confirmed by flow cytometry by using an ALV-B surface

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This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: MLV, murine leukemia virus; ALV, avian leukosis virus; TVB, subgroup B ALV receptor; SU, surface envelope protein; EGF, epidermal growth factor; EGFR, EGF receptor; lacZ,  $\beta$ -galactosidase; GFP, green fluorescent protein.

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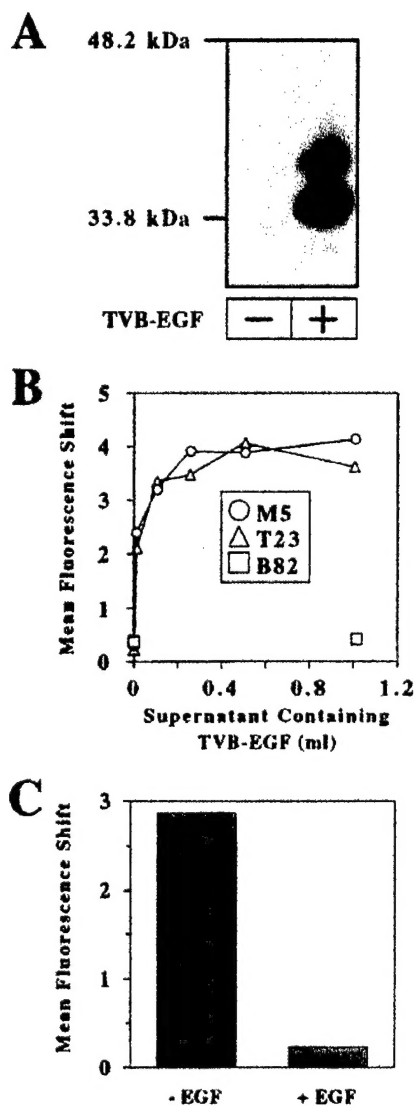


FIG. 1. TVB-EGF binds specifically to cell surface EGFR. (A) Aliquots of extracellular supernatants from nontransfected 293 cells (–) and transfected 293 cells expressing TVB-EGF (+) were subjected to SDS/PAGE and to immunoblotting by using an EGF-specific antibody. (B) Mouse M5 cells expressing kinase-deficient EGFR, T23 cells expressing wild-type EGFR, and B82 cells, which lack EGFR, were incubated with increasing amounts of TVB-EGF. (C) M5 cells were incubated with TVB-EGF in the presence or absence of a recombinant EGF protein. (B–C) After each of these incubations, the cells were incubated with ALV-B SU-Ig and a FITC-conjugated secondary antibody and analyzed by flow cytometry as described (20).

envelope protein (SU)-Ig and a FITC-conjugated secondary antibody as described (26).

**Production of TVB-EGF.** TVB-EGF was produced in the extracellular supernatants of transiently transfected 293 cells as described (20), and 50- $\mu$ l aliquots of extracellular supernatants from transfected and nontransfected 293 cells were subjected to electrophoresis on a 12% polyacrylamide gel containing SDS and transferred to a nylon membrane. The samples were subjected to immunoblotting by using an EGF-specific antibody (PeproTech, Rocky Hill, NJ) diluted 1:500 in TBST (25 mM Tris, pH 7.4/130 mM NaCl/0.1% Triton X-100) followed by a horseradish peroxidase-coupled antibody specific for rabbit IgG (Amersham Pharmacia) diluted 1:3,000 in TBST. The bound antibodies were detected by enhanced chemiluminescence.

**Flow Cytometry.** Flow cytometric analysis to detect TVB-EGF binding to cells that express EGFR was performed on ice essentially as described (20). B82, M5, and T23 cells were incubated with different amounts of TVB-EGF-containing supernatants, and M5 cells were incubated with a saturating amount of TVB-EGF (100  $\mu$ l of crude extracellular supernatant in 500  $\mu$ l of medium) in the presence or absence of 4  $\mu$ g of recombinant EGF (Upstate Biotechnology, Lake Placid, NY). The bound TVB-EGF proteins were detected by adding 5  $\mu$ g of ALV-B SU-Ig and 5  $\mu$ g of an FITC-coupled anti-rabbit secondary antibody (Dako).

**Production of Viral Pseudotypes.** MLV-lacZ (ALV-B) virions were generated by using a tripartite transfection system (27). Typically, a 60% confluent plate of 293 cells was transfected with 15  $\mu$ g of plasmid pMD.ori.gag.pol, 20  $\mu$ g of plasmid pMMP-nlslacZ, and 5  $\mu$ g of plasmid pAB7 encoding ALV-B Env. Virions contained in extracellular supernatants were harvested at approximately 48 and 60 h after transfection and pooled. These virions were used for infection studies either before or after being concentrated by ultracentrifugation as described (28). To generate preloaded virions, MLV-lacZ (ALV-B) was incubated at either a 1:1 or a 1:2 ratio with extracellular supernatants containing TVB-EGF, and the virion/bridge-protein complexes were purified by ultracentrifugation at  $109,000 \times g$  for 1.5 h at 4°C. The MLV-lacZ (ALV-B)/TVB-EGF complexes were resuspended in TNE buffer (described in ref. 28; at 1/100, 1/50, or 1/33 of the original volume) to generate 100 $\times$ , 50 $\times$ , or 33 $\times$  stocks of preloaded virions).

To generate preloaded virions directly from virus packaging cells, 293 cells were transiently transfected essentially as described above, except that variable amounts of pAB1 plasmid DNA were cotransfected. To monitor expression levels of TVB-EGF, 40- $\mu$ l aliquots of extracellular supernatants prepared from these cells were subjected to electrophoresis on a 10% polyacrylamide gel containing SDS and transferred to a nylon membrane. These samples were immunoblotted with 3 ml of extracellular supernatants containing a subgroup B ALV SU-Ig fusion protein (22) diluted in 10 ml of TBST followed by an horseradish peroxidase-coupled antibody specific for rabbit IgG (Amersham Pharmacia) diluted 1:3,000 in TBST. Virions produced from cells transfected with 0.01  $\mu$ g, 0.1  $\mu$ g, or 1  $\mu$ g of pAB1 plasmid DNA were collected 48 h after transfection and either left unconcentrated or concentrated 100-fold before infection. Human 293 cells transiently producing MLV-GFP/TVB-EGF complexes were generated as described above except that plasmid pCMMP.GFP/neo (J.-S. Lee and R. C. Mulligan, unpublished work) was used instead of pMMP-nlslacZ.

**Infection of Cells Preloaded with TVB-EGF.** M5 cells plated at 20% confluence in six-well tissue culture plates were placed on ice and incubated for 1 h at 4°C with 1 ml of ice-cold extracellular supernatants that either contained or lacked TVB-EGF. The cells were then washed with ice-cold PBS and incubated for 1 h at 4°C with 1.5 ml of ice-cold medium containing 5  $\mu$ g/ml polybrene and 0.75  $\mu$ l of (100 $\times$ ) MLV-lacZ (ALV-B). The M5:S3/T cells were also incubated with

Table 1. TVB-EGF mediates efficient viral entry when attached to M5 cells

Cell line	Viral titer per microliter
M5:S3/T	$(1.3 \pm 0.2) \times 10^3$
M5 (+TVB-EGF)	$(7.1 \pm 2.4) \times 10^2$
M5 (–TVB-EGF)	0

M5 cells were incubated with extracellular supernatants that contained (+) or lacked (–) TVB-EGF. These cells, and for control purposes M5:S3/T cells, were then challenged with 0.75- $\mu$ l aliquots of (100 $\times$ ) concentrated MLV-lacZ (ALV-B), and the infected cells were identified by analyzing for lacZ expression.

the same amount of virus under identical conditions. The cells were transferred to a 37°C incubator and, 52 h later, were washed with PBS and fixed for 15 min at room temperature with 1% formaldehyde/0.2% glutaraldehyde in PBS. The cells were then washed twice with PBS and incubated with 2 mM MgCl<sub>2</sub>/5 mM potassium ferrocyanide/5 mM potassium ferricyanide/0.01 g of 5-bromo-4-chloro-3-indolyl β-D-galactoside (GIBCO)/1% *N,N*-dimethylformamide (Mallinckrodt) in PBS to detect the viral-encoded lacZ protein.

**Infection of Cells with Virions Preloaded with TVB-EGF.** Cells were plated at approximately 20% confluence on 12-well tissue culture plates before viral challenge. M5, B82, and T23 cells were incubated with 190 μl of (33×) MLV-lacZ (ALV-B), and M5:S3/T cells were incubated with 0.1 μl of these concentrated virions. M5, T23, and B82 cells were incubated separately with 0.5 μl, 1 μl, and 200 μl of (33×) MLV-lacZ (ALV-B)/TVB-EGF complexes, respectively. The cells were immediately placed at 37°C and, approximately 60 h later, were analyzed for infection by staining for lacZ activity as before.

To analyze virion thermostability, 0.5 μl of (50×) MLV-lacZ (ALV-B) or 2 μl of (50×) MLV-lacZ (ALV-B)/TVB-EGF complexes were incubated either on ice for 9 h (preincubation time = 0) or at 37°C for the time periods indicated and then returned to ice before addition to M5:S3/T cells or to M5 cells, respectively.

In the cocultivation experiment, human 293 cells producing MLV-GFP (ALV-B)/TVB-EGF complexes were placed, 16 h after transfection, in transwell inserts with a 0.4-μm pore size (CoStar). These transwell inserts were placed in six-well tissue culture plates that contained either M5 or B82 cells plated at approximately 20% confluence and incubated at 37°C. The transwell inserts were removed 56 h after the start of cocultivation, and the numbers of infected cells were determined by using flow cytometry to detect expression of GFP.

## RESULTS

**TVB-EGF Is a Bifunctional Reagent That Can Bind Both to Cell Surface EGF Receptors and to ALV-B SU.** The TVB-EGF protein is comprised of the extracellular domain of the TVBS3 receptor for subgroups B and D ALV (ALV-B and ALV-D; refs. 22 and 29), fused via a glycine- and proline-rich linker region to the mature form of human EGF (30). TVB-EGF was produced in the extracellular supernatants of transiently transfected human 293 cells, and its production was confirmed by immunoblotting by using an EGF-specific antibody (Fig. 1A). Three predominant forms of TVB-EGF were detected (Fig. 1A), presumably resulting from differential glycosylation of the TVB domain as noted previously (22); the expected molecular mass of TVB-EGF without any N-linked carbohydrate residues is approximately 22 kDa.

Flow cytometric analysis was used to assess whether TVB-EGF is a bifunctional reagent that can bind simultaneously to cell surface EGF receptors and to the ALV-B SU subunit that is responsible for TVB binding. Cells were incubated with increasing amounts of supernatants that contained TVB-EGF and then incubated with a subgroup B-specific ALV SU-Ig fusion protein (22) followed by an FITC-conjugated secondary antibody. TVB-EGF bound in a saturable manner to transfected mouse L cells expressing kinase-deficient human EGFR (M5 cells) and to transfected mouse L cells expressing wild-type human EGFR (T23 cells; Fig. 1B). By contrast, the bridge protein did not bind to the parental mouse L cells, which lack EGF receptors (B82 cells), even at the highest amount of TVB-EGF added (Fig. 1B). In addition, TVB-EGF binding to M5 cells was specifically competed in the presence of recombinant EGF (Fig. 1C). Taken together, these data show that TVB-EGF binds specifically to ALV-B SU and to the ligand-binding regions of EGFR expressed on the surfaces of M5 and T23 cells.

**TVB-EGF Mediates Viral Infection when Attached to Cell-Surface EGFR.** To determine whether TVB-EGF can facilitate viral entry when attached to cell surface EGFR, we asked whether this bridge protein could mediate infection by an MLV vector encoding lacZ and bearing ALV-B Env [designated MLV-lacZ (ALV-B)]. An MLV vector was chosen for this study, because, to date, these are the best characterized retroviral vectors used for infecting dividing cell types such as cancer cells (31). As expected, the MLV-lacZ (ALV-B) pseudotyped virus required cell surface TVB receptors for entry; in contrast to parental M5 cells, which were resistant to viral infection, transfected M5 cells (M5:S3/T) expressing a transmembrane form of TVBS3 were susceptible to infection by MLV-lacZ (ALV-B) (Table 1).

To assess whether TVB-EGF could mediate viral entry when bound to M5 cells, these cells were incubated with the bridge protein before challenge with MLV-lacZ (ALV-B). TVB-EGF rendered these cells highly susceptible to viral infection; the level of infection mediated by TVB-EGF was approximately 55% of that seen when control M5:S3/T cells were challenged with the same number of native virions (Table 1). M5 cells that were incubated with TVB-EGF were also very susceptible to infection by subgroup B-specific ALV-based vectors (data not shown). These results establish that TVB-EGF is a highly efficient mediator of targeted retroviral entry when attached to cell surface EGFR before viral challenge.

**Virions Preloaded with TVB-EGF Specifically Infect Cells That Express EGFR.** For most retroviral delivery applications, it would be desirable to preload the bridge protein onto virions, as opposed to cell surfaces, provided that such viral complexes retain their cell-type specificity. To determine whether retroviruses preloaded with TVB-EGF have this property, MLV-lacZ (ALV-B) virions were incubated with the bridge protein, and the resultant complexes were purified and used to infect M5, T23, and B82 cells.

The level of preloaded virus infection observed with M5 cells was extremely high and, in fact, exceeded the level obtained when the same number of native virions were used to infect the control M5:S3/T cells (Table 2). The level of preloaded virus infection of T23 cells was also high, representing approximately 29% of the level seen with native virus infection of M5:S3/T cells (Table 2). By contrast, the level of preloaded virus infection of B82 cells was low (Table 2). Indeed, similar low levels of infection were seen with B82, M5, and T23 cells that were challenged with native virions (Table 2), showing that these events are caused by an intrinsic ability of MLV-lacZ (ALV-B) to enter these cell types with low efficiency. Based on a comparison of the numbers of infection events obtained with cells that express or lack EGFR (Table 2), it seems that 99.97% of the preloaded virus infection events seen with M5 cells and 99.89% of those seen with T23 cells require the specific interaction of the virus-bound TVB-EGF and cell-surface EGFR.

Table 2. MLV-lacZ (ALV-B) virions preloaded with TVB-EGF specifically infect M5 and T23 cells with a high efficiency

Cell line	TVB-EGF	Viral titer per milliliter
B82	—	$(6.6 \pm 0.9) \times 10^2$
	+	$(5.0 \pm 0.2) \times 10^2$
M5	—	$(8.8 \pm 1.2) \times 10^2$
	+	$(2.2 \pm 0.2) \times 10^6$
T23	—	$(2.1 \pm 0.2) \times 10^2$
	+	$(4.5 \pm 0.4) \times 10^5$
M5:S3/T	—	$(1.6 \pm 0.04) \times 10^6$

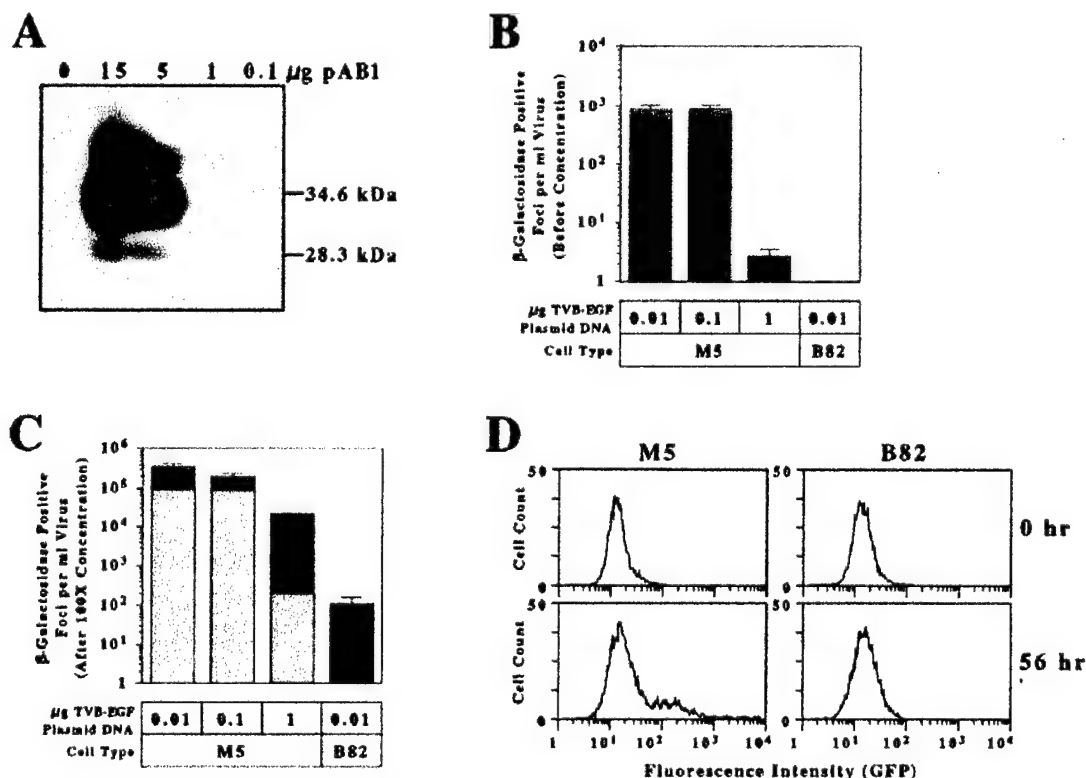
B82, M5, and T23 cells were challenged with MLV-lacZ (ALV-B) and MLV-lacZ (ALV-B)/TVB-EGF complexes. M5:S3/T cells were also challenged with MLV-lacZ (ALV-B). Infected cells were identified by staining for lacZ activity.

**Preloaded Virions Can Be Generated Directly from Virus Packaging Cells.** To determine whether virions preloaded with TVB-EGF could be produced from virus packaging cells, human 293 cells were cotransfected with plasmids encoding MLV-lacZ (ALV-B) and with different amounts of the pAB1 plasmid encoding TVB-EGF. The addition of increasing amounts of the pAB1 plasmid led to an increase in the production of TVB-EGF from the virus packaging cells (Fig. 2A). Virion/TVB-EGF complexes contained in the extracellular supernatants from virus-producing cells that were transfected with 0.01  $\mu$ g, 0.1  $\mu$ g, or 1  $\mu$ g of pAB1 plasmid DNA were able to infect M5 cells but not B82 cells (Fig. 2B). However, the level of infection of M5 cells was substantially reduced when 1  $\mu$ g of pAB1 plasmid DNA was transfected (Fig. 2B). Given the relationship between the amount of pAB1 plasmid DNA transfected and the amount of TVB-EGF produced (Fig. 2A), we reasoned that this inhibitory effect might be caused by the production of excess levels of the bridge protein, which might act as a competitive inhibitor of preloaded virus entry. To test this possibility, virion/TVB-EGF complexes were purified from unbound bridge protein in the extracellular supernatants by ultracentrifugation and were concentrated 100-fold before infection of M5 and B82 cells. With M5 cells, the infectious titers obtained with these complexes purified from cells transfected with 0.01  $\mu$ g and 0.1  $\mu$ g of pAB1 plasmid DNA were similar to those expected from the 100-fold concentration effect (Fig. 2C). Indeed, it is noteworthy that, by using 0.01  $\mu$ g of pAB1 plasmid DNA, the purified preloaded

virions infected M5 cells 3,300-fold more efficiently than B82 cells, which lack EGFR (Fig. 2C). This level is consistent with the specificity of infection obtained when TVB-EGF was preloaded onto native virions (Table 2). By contrast, the titer obtained with virions purified from cells transfected with 1  $\mu$ g of pAB1 plasmid DNA was approximately 45-fold higher than that expected from the concentration effect alone (Fig. 2C), consistent with the idea that excess amounts of the bridge protein must be removed for efficient infection by these preloaded viruses.

As an independent test of the specificity of the preloaded virions, virus packaging cells were generated that produced TVB-EGF and MLV (ALV-B) particles encoding GFP. These cells were cocultivated with either M5 or B82 cells, and the target cells were analyzed for infection by flow cytometry. After 56 h of cocultivation, approximately 20% of the M5 cells were infected by virus (Fig. 2D Left). In contrast to M5 cells, there was no discernable infection of B82 cells under the same conditions (Fig. 2D Right). These results underscore the strict cell-type-specific infection of the preloaded virions.

The fact that preloaded virions were infectious, even when produced from virus packaging cells, indicated that these viral complexes must be somewhat stable at 37°C. To investigate their thermostability, preloaded viruses were incubated at 37°C for different periods of time before addition to M5 cells. For control purposes, these experiments were also performed by using native virions that were then used to infect the control M5:S3/T cells. These studies showed that both the native



**FIG. 2.** TVB-EGF-loaded virions produced directly from virus packaging cells infect M5 cells specifically. Human 293 cells were transfected with fixed amounts of plasmids encoding MLV-lacZ (ALV-B) and with variable amounts of plasmid pAB1 encoding TVB-EGF. (A) Aliquots of extracellular supernatants taken from these cells were monitored for TVB-EGF production by immunoblotting analysis by using a subgroup B ALV SU-Ig fusion protein (22) and a horseradish peroxidase-coupled secondary antibody. (B) M5 and B82 cells were challenged with MLV-lacZ (ALV-B) virions produced from cells that were cotransfected with the amounts of pAB1 plasmid DNA indicated, and the resultant numbers of infected cells were measured by lacZ staining. (C) MLV-lacZ (ALV-B)/TVB-EGF complexes shown in B were purified from unbound TVB-EGF and concentrated 100-fold before infection. Gray bars represent the expected infectious viral titer based on the 100-fold concentration effect (as compared with those numbers obtained in B). Black bars represent the actual infectious titers observed with the purified virion/TVB-EGF complexes. (D) M5 cells or B82 cells were cocultivated with human 293 cells that produce MLV-GFP (ALV-B)/TVB-EGF complexes. Flow cytometric profiles for M5 (Left) and B82 (Right) are shown after 0 or 56 h of cocultivation.

virions (Fig. 3A) and preloaded virions (Fig. 3B) were inactivated when incubated for several hours at 37°C. It is clear from these data, however, that the preloaded virions are at least as stable as native virions when preincubated at 37°C for a short time period.

### DISCUSSION

In this report, we have shown that TVB-EGF, a model TVB-ligand bridge protein, can be used to target infection by retroviral vectors to cells that express EGFR. TVB-EGF that was bound to M5 cell surface EGFR before viral challenge efficiently mediated infection by a pseudotyped MLV vector bearing the ALV-B Env protein, whereas there was no obvious infection of B82 cells, which lack EGFR. Indeed, the level of infection obtained with M5 cells was approximately one-half of that seen with control TVB-expressing M5 cells challenged with the same number of native virions.

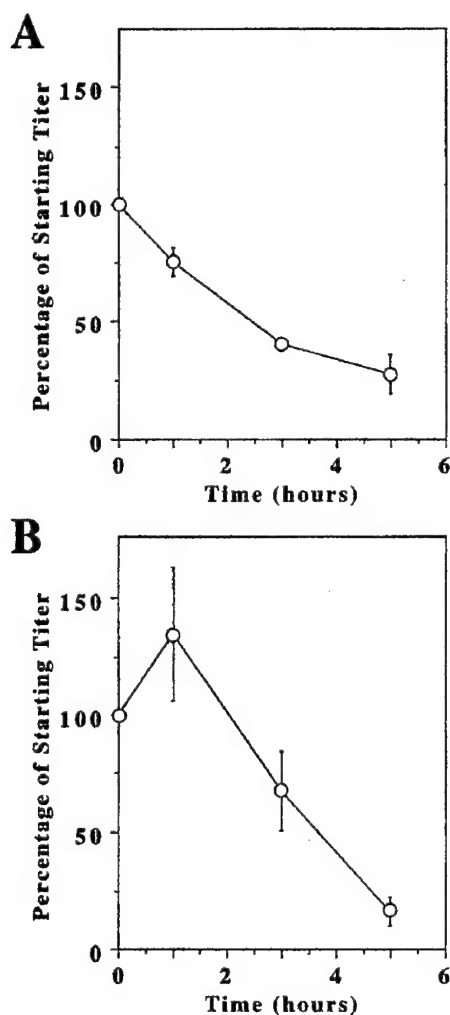


FIG. 3. MLV-lacZ (ALV-B)/TVB-EGF complexes are stable at 37°C. MLV-lacZ (ALV-B) (A) or MLV-lacZ (ALV-B Env)/TVB-EGF (B) complexes were incubated at 37°C for different time intervals before addition to M5:S3/T cells or to M5 cells, respectively. The number of infected cells was measured by lacZ staining. The results shown reflect the percentage remaining of the starting virus titer (at  $t = 0$ ). The starting titer of MLV-lacZ (ALV-B) was approximately  $2 \times 10^5$  lacZ-positive colonies per ml and that of the virion/TVB-EGF complexes was approximately  $3 \times 10^4$  lacZ-positive colonies per ml.

The most important activity of TVB-EGF was that it also supported efficient viral entry when attached to virions before their addition to cells. These preloaded viruses were produced successfully either by mixing native virions with extracellular supernatants containing TVB-EGF or instead by coexpressing the bridge protein in virus packaging cells. Virions preloaded with TVB-EGF specifically infected M5 and T23 cells with an efficiency that exceeded or closely approximated that seen with control TVB-expressing M5 cells challenged with native virions. In these experiments, the preloaded virions preferentially infected M5 cells as compared with T23 cells (Table 2). The reason for this difference between the two cell types is currently under investigation and might be linked to differences in rates of endocytosis and/or trafficking of kinase-deficient EGFR, as opposed to wild-type forms of EGFR, as discussed previously (20).

In contrast to M5 cells and T23 cells, B82 cells were only infected at a low level by virions preloaded with TVB-EGF. It is extremely unlikely that this small number of infection events was caused by the fact that these virions were preloaded with the retroviral receptor-ligand bridge protein before addition to cells, because similar numbers of infected cells were seen when B82, T23, and M5 cells were challenged with native virions. Instead, it seems that this low level of nonspecific infection is caused by an intrinsic ability of the MLV pseudotyped viruses to enter these cells with a low efficiency.

An unexpected finding of these studies was that virions bound to TVB-EGF were at least as stable as native virions when incubated at 37°C before addition to cells. Based on previous studies of ALV-A Env/TVA interactions, we had expected that MLV-lacZ (ALV-B) pseudotypes preloaded with TVB-EGF might be unstable under these conditions, because TVA binding induces rapid temperature-dependent conformational changes in a soluble ALV-A Env protein that are similar to those expected to give rise to the fusion-active form of the viral glycoprotein (32, 33). In the absence of an appropriate target cell membrane, these receptor-induced structural changes in Env might be expected to inactivate the viral glycoprotein in much the same way seen with low-pH-treated influenza virus hemagglutinin glycoproteins (34). Consistent with this idea, we have not been able to efficiently infect M5 cells with MLV-lacZ (ALV-A) vectors that are preloaded with TVA-EGF (data not shown). Although the precise defect of these TVA-EGF-loaded viruses remains to be established, these data indicate an apparent difference between ALV-A and ALV-B Env-receptor interactions and show that these types of bridge proteins can be useful tools for studying parameters that influence the efficiency of retroviral entry in addition to their use as viral targeting reagents.

Another important result obtained from these studies was that TVB-EGF-loaded MLV vectors bearing ALV-B Env can be stably produced from virus packaging cells while retaining their targeting specificity. This result opens up the possibility of targeting a variety of different retroviral vectors (e.g., those based on ALV, MLV, or lentiviruses) by simply expressing both ALV-B Env and a TVB-ligand bridge protein in cells that produce these viral vectors. This possibility, in turn, could have important implications for the delivery of retroviral vectors to specific cell types *in vivo*. Previously, it was shown that infection of cancer cells *in vivo* by MLV vectors could be achieved by direct injection of virus packaging cells (35). The approach described in this study could extend the specificity of infection that can be achieved by this method by introducing cells that produce viral vectors that are preloaded with TVB-ligand bridge proteins with defined target cell specificities.

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## A TVA–Single-Chain Antibody Fusion Protein Mediates Specific Targeting of a Subgroup A Avian Leukosis Virus Vector to Cells Expressing a Tumor-Specific Form of Epidermal Growth Factor Receptor

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**We have previously described an approach that employs retroviral receptor-ligand bridge proteins to target retroviral vectors to specific cell types. To determine whether targeted retroviral entry can also be achieved using a retroviral receptor–single-chain antibody bridge protein, the TVA-MR1 fusion protein was generated. TVA-MR1 is comprised of the extracellular domain of the TVA receptor for subgroup A avian leukosis viruses (ALV-A), fused to the MR1 single-chain antibody that binds specifically to EGFRvIII, a tumor-specific form of the epidermal growth factor receptor. We show that TVA-MR1 binds specifically to a murine version of EGFRvIII and promotes ALV-A entry selectively into cells that express this cell surface marker. These studies demonstrate that it is possible to target retroviral vectors to specific cell types through the use of a retroviral receptor–single-chain antibody fusion protein.**

Several different strategies have been developed for targeting infection of specific cell types by retroviral vectors. The most common approach has employed recombinant viral envelope (Env) proteins that contain either cell type-specific ligands or single-chain antibodies that bind to specific cell surface molecules (1, 4, 7–12, 15, 18–20, 24–28, 31–35, 38, 39, 41, 42, 44, 46, 48, 51–54, 57). This approach requires that the specific alterations made to Env do not affect the biosynthesis, virion assembly, or fusogenic function of the viral glycoprotein (12, 54, 57).

An alternative approach for targeting retroviral entry that employs soluble retroviral receptor-ligand bridge proteins was recently developed (5, 47). These bridge proteins are bifunctional reagents: the ligand moiety binds to specific cell surface receptors and the retroviral receptor moiety binds to Env, activating viral entry. This technique does not require making alterations to the viral glycoprotein but instead relies on “wild-type” Env-receptor interactions to target viral entry.

To demonstrate the feasibility of this approach, the TVA-EGF and TVB-EGF fusion proteins were generated. These bridge proteins contained human epidermal growth factor (EGF) fused to the extracellular domains of either the TVA receptor for subgroup A avian leukosis viruses (ALV-A) or the TVB receptor for ALV-B and ALV-D, respectively. Each of these bridge proteins promoted specific retroviral entry into cells that express the EGF receptor (EGFR) when bound to cell surfaces before viral challenge (5, 47). Furthermore, murine leukemia virus (MLV) pseudotypes bearing ALV-B Env (EnvB) and preloaded with TVB-EGF were targeted specifically to cells that express EGFR (5). These data demonstrated

that retroviral vectors could be targeted to specific cell types by binding retroviral receptor-ligand bridge proteins to virions or to cell surfaces before viral challenge.

To extend the utility of this approach, we have now asked whether targeted retroviral entry into cells can also be achieved using TVA-MR1, a bridge protein that contains the extracellular domain of TVA fused to the MR1 single-chain antibody. This antibody binds specifically to the extracellular region of EGFRvIII, a variant form of the EGFR that is expressed at the surface of human tumor cells, including those derived from lung and breast carcinomas and glioblastomas (14, 17, 30, 37, 40, 49, 55, 56). EGFRvIII lacks a substantial portion of the extracellular domain of the wild-type receptor as a consequence of a deletion or rearrangement that commonly occurs when the EGFR gene is amplified during tumor biogenesis. These alterations result in constitutive, ligand-independent activation of EGFRvIII (22), which, in turn, confers a transformed phenotype upon various cell lines (22, 40).

The MR1 antibody binds to a novel polypeptide sequence that is formed at the site of the deletion or rearrangement that gives rise to EGFRvIII (29). The combination of the tumor-restricted expression of EGFRvIII and the binding specificity of the MR1 antibody makes this an attractive model system to test whether retroviral receptor–single-chain antibody bridge proteins can mediate targeted viral entry into cells. In this report, we demonstrate that TVA-MR1 can support efficient and specific ALV entry into mammalian cells that have been engineered to express a murine form of EGFRvIII.

### MATERIALS AND METHODS

**Viruses and immunoadhesins.** The SUA-rIgG immunoadhesin was described elsewhere and is comprised of the surface protein of the Schmidt-Ruppin A strain of Rous sarcoma virus fused to a rabbit Fc chain (58). ALV-A-specific vectors encoding the enhanced green fluorescent protein (EGFP; Clontech) were generated by transfection of DF1 cells (45) with the RCASBP(A)-EGFP plasmid (provided by Mark Federspiel and Matt van Brocklin). The transfected

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cells were propagated until 100% of the population expressed EGFP as determined by fluorescence microscopy. Cells were then seeded in 1,700-cm<sup>2</sup> roller bottles, and virions were harvested every 12 h in 50 ml of medium that was equilibrated with 5% CO<sub>2</sub>. This medium was pooled and filtered through 0.45- $\mu$ m-pore-size filters and stored at -80°C. Before use, the virus-containing supernatants were thawed and subjected to centrifugation at 109,000  $\times$  g for 1.5 h at 4°C. The viral pellets were then resuspended overnight at 4°C in 1/100 of the original volume of TNE buffer (5).

Replication-defective MLV vectors encoding a synthetic transmembrane form of TVA (TVA<sup>TM</sup>) (3) or a murine form of EGFRvIII were generated. The MLV vector pMMP.TVA<sup>TM</sup> was generated by first preparing a DNA fragment that encodes TVA<sup>TM</sup> by PCR amplification using pDW1 plasmid template DNA (D. Wenzke and J. A. T. Young, unpublished data) and the following two primers: 5'-GCATAGCGTACCATGGCTAGATTGCTTCTGCGATTGC-3' and 5'-CGATCGACATGCATCCGGAAGTAATCGATCTGAGCAGCGTAATCTGG-3'. The resultant DNA fragment was digested with *Nco*I and *Bsp*EI restriction enzymes and was subcloned into the *Nco*I and *Bsp*EI sites of the pMMP.EGFP plasmid (K. Bradley and J. A. T. Young, unpublished data), generating the pMMP.TVA<sup>TM</sup> plasmid. The MLV vector pSFG.EGFRvIII contains a gene encoding a murine form of EGFRvIII (R. Carter and R. C. Mulligan, unpublished data) located between the *Nco*I and *Bam*HI restriction enzyme sites of the pSFG vector.

Stocks of MLV vectors pseudotyped with the VSV-G protein were prepared from human 293T cells by using a transient transfection system essentially as described previously (5) with 5  $\mu$ g of the pMD.G plasmid encoding VSV-G, 15  $\mu$ g of the pMD.ori.gagpol plasmid encoding MLV Gag and Pol, and 15  $\mu$ g of either plasmid pSFG.EGFRvIII or plasmid pMMP.TVA<sup>TM</sup>. Virions were harvested at 48 and 72 h postinfection, and the supernatants containing MLV-EGFRvIII(VSV-G) and MLV-TVA<sup>TM</sup>(VSV-G) viruses were separately pooled and filtered through a 0.45- $\mu$ m-pore-size filter and then stored at -80°C.

**Cell lines.** Human 293T cells were propagated in Dulbecco modified Eagle medium containing 5% fetal bovine serum. 293T-EGFRvIII cells were generated by transducing 293T cells with MLV-EGFRvIII(VSV-G). Approximately 72 h after viral challenge, the transduced cell population (10<sup>7</sup> cells) was incubated for 30 min at 4°C with 5 ml of extracellular supernatant containing TVA-MR1 and then with 5 ml of extracellular supernatant containing SUA-rIgG. These cells were then incubated for 10 min at 4°C with a fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit antibody (DAKO Corp.) diluted 1:100 in medium. The cells were washed with ice-cold medium, and those expressing the EGFRvIII protein were isolated by flow cytometric sorting using a Coulter Epics Elite cell sorter. 293T-TVA<sup>TM</sup> cells were generated in a similar manner, except that 293T cells were transduced with MLV-TVA<sup>TM</sup>(VSV-G). Cells expressing the TVA<sup>TM</sup> receptor were isolated by flow cytometric sorting after binding SUA-rIgG to the cells for 30 min at 4°C, followed by binding the FITC-conjugated anti-rabbit antibody; 25% of these cells with the highest level of cell surface TVA<sup>TM</sup> were then isolated by subjecting the population to an additional round of flow cytometric sorting.

**Construction and expression of TVA-MR1.** A DNA fragment encoding the MR1 single-chain antibody (29) was generated by PCR amplification, using as template DNA the plasmid pCMMF.MR1, which contains a gene encoding MR1 located between the *Xba*I and *Hind*III restriction enzyme sites of the pCMMF vector (T. Niederman et al., unpublished data), and the following two oligonucleotide primers: 5'-GAAGCTCTAGGGGGACCGCAGGTACAACCTCCAGCAGTCCGGGGG-3' and 5'-GAGGGGGCCCTAGATTATAGAGCTTTTTC AAGCTTGGTCCATCACCG-3'. The resultant DNA fragment was digested with *Apa*I, and repaired with the Klenow fragment of DNA polymerase, and digested with *Avr*II to generate a 758-bp DNA fragment encoding MR1. This fragment was then ligated with plasmid pSS8 that had been cut with *Asp*718, and repaired, and then digested with *Avr*II. Plasmid pSS8 contains the gene encoding a TVA-hergulin $\beta$ 1 fusion protein (S. Snitkovsky and J. Young, unpublished data) located between the *Eco*RI and *Hpa*I restriction enzyme sites of the pCI expression vector (Promega). The resultant plasmid pSS11 encodes the TVA-MR1 fusion protein, and the authenticity of this open reading frame was confirmed by DNA sequence analysis (performed by the core DNA sequencing facility in the Department of Microbiology and Molecular Genetics at Harvard Medical School).

To generate the TVA-MR1 fusion protein, plasmid pSS11 was transfected into human 293 cells as described previously (47). Aliquots of 40  $\mu$ l of extracellular supernatant taken from transfected and nontransfected human 293 cells were subjected to electrophoresis on 10% polyacrylamide gel containing sodium dodecyl sulfate under nonreducing conditions. The proteins were then transferred to a nitrocellulose membrane, and TVA-MR1 was detected by immunoblotting with 10 ml of extracellular supernatant containing SUA-rIgG and then with a horseradish peroxidase-conjugated antibody specific for rabbit immunoglobulins (Amersham), followed by enhanced chemiluminescence.

**TVA-MR1 binding studies.** A suspension of 3.5  $\times$  10<sup>8</sup> 293T-EGFRvIII cells was incubated for 1 h at 4°C with increasing amounts of TVA-MR1-containing extracellular supernatant. These samples were made up to a total volume of 500  $\mu$ l with 293 cell-conditioned medium (medium that was obtained from a confluent monolayer of human 293 cells). A suspension of 3.5  $\times$  10<sup>8</sup> 293T cells was incubated for 1 h at 4°C with 500  $\mu$ l of extracellular supernatant that either lacked or contained TVA-MR1. Both cell populations were then washed with 2

ml of ice-cold phosphate-buffered saline (PBS) and incubated for 1 h at 4°C with 500  $\mu$ l of extracellular supernatant containing SUA-rIgG. The cells were washed again with ice-cold PBS and incubated for 30 min at 4°C with the FITC-conjugated swine anti-rabbit antibody (diluted 1:100) in medium. The cells were then washed again and resuspended in 500  $\mu$ l of ice-cold PBS containing 1% formaldehyde, and the bound TVA-MR1 fusion proteins were detected by flow cytometry using a Coulter Epics Ex-L instrument.

The peptide competition binding experiments were performed by incubating 500  $\mu$ l of extracellular supernatant containing TVA-MR1 for 1 h at 4°C with a final 100 nM concentration of either the MR1 epitope-containing peptide (LEKKGNVVDTH) (29) or a scrambled (control) version of this peptide (YKELGVEVDNKHT). These samples were then incubated with 293T-EGFRvIII cells for 1 h at 4°C. TVA-MR1 proteins that were bound to the cells were then detected using SUA-rIgG and the FITC-conjugated antibody as described above. For control purposes, 500  $\mu$ l of 293 cell supernatants containing a 100 nM concentration of either the MR1 epitope-containing peptide or of the control peptide was placed on ice for 1 h and then incubated with 293T-TVA<sup>TM</sup> cells for an additional hour. The cells were then washed with ice-cold PBS, and cell surface TVA<sup>TM</sup> proteins were detected by flow cytometry as described above.

**TVA-MR1-mediated infection.** Each of the following steps was performed at 4°C, with a suspension of approximately 5  $\times$  10<sup>5</sup> cells of each cell type, and each experiment was performed in triplicate. 293T cells were rocked together for 1 h with 500  $\mu$ l of 293 cell-conditioned medium that either lacked or contained TVA-MR1. 293T-EGFRvIII cells were similarly incubated with 500  $\mu$ l of extracellular supernatant containing TVA-MR1. 293T-TVA<sup>TM</sup> cells were incubated with 500  $\mu$ l of 293 cell-conditioned medium for 1 h.

Following each of these incubations, the cells were washed and resuspended in 500  $\mu$ l of medium with or without 5  $\mu$ l of 100-fold-concentrated RCASBP(A)-EGFP (0.5  $\mu$ l of 100-fold-concentrated virus for infection of 293T-TVA<sup>TM</sup> cells). The cells were then rocked with virus for 1 h at 4°C before plating and incubation at 37°C. The medium was replaced 20 h later; 72 h after viral challenge, the cells were resuspended in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS containing 1 mM EDTA and 7  $\mu$ M propidium iodide and were analyzed for EGFP expression using a Coulter Epics Ex-L flow cytometer. Dead cells that had taken up propidium iodide were excluded from this analysis by electronic gating. The viral titer was then measured by first calculating the multiplicity of infection (MOI) observed from the input virus: MOI = -ln[1 - (number of EGFP fluorescent cells/total number of cells analyzed)]. The actual titers (per microliter of 100-fold-concentrated virus) were then calculated using the following equation: (MOI  $\times$  total number of cells that were challenged/5 (or divided by 0.5 in the case of 293T-TVA<sup>TM</sup> cells). The number of fluorescent cells seen in the absence of virus was subtracted from each of the calculations.

Peptide competition experiments were performed by incubating 500  $\mu$ l of extracellular supernatant containing TVA-MR1 at 4°C for 1 h with a 100 nM concentration of either the MR1 epitope-containing peptide or of the control peptide. These samples were then incubated with 293T-EGFRvIII cells for another hour at 4°C. The cells were then washed and incubated for 1 h with 500  $\mu$ l of medium containing 5  $\mu$ l of 100-fold-concentrated RCASBP(A)-EGFP before plating and incubation at 37°C for 18 h. The medium was then replaced, and 72 h after viral challenge, the cells were analyzed by flow cytometry as described above.

## RESULTS

**Production of the TVA-MR1 protein.** A gene that encodes the recombinant TVA-MR1 bridge protein was constructed in plasmid pSS11 (described in Materials and Methods). TVA-MR1 is comprised of the extracellular portion of the TVA receptor (2) fused to the N terminus of the MR1 single-chain antibody, which recognizes a unique sequence in the extracellular domain of EGFRvIII (29) (Fig. 1A). A proline-rich polypeptide linker that was described previously (47) was inserted between each of the two domains of TVA-MR1 (Fig. 1A) in order to maximize the probability that each domain would fold and function independently. TVA-MR1 was produced as a secreted protein, approximately 54 kDa in size, in the extracellular supernatants taken from human 293 cells transfected with plasmid pSS11 (Fig. 1B, compare lanes 1 and 2).

**TVA-MR1 specifically binds to cells expressing an EGFRvIII protein.** To determine whether the Env-binding and antigen-binding domains of TVA-MR1 can each function independently, we investigated whether this bridge protein can bind both to the ALV-A surface envelope (ALV-A SU) and to a cell surface EGFRvIII protein. Transfected human 293T cells expressing a murine form of EGFRvIII (293T-EGFRvIII cells)

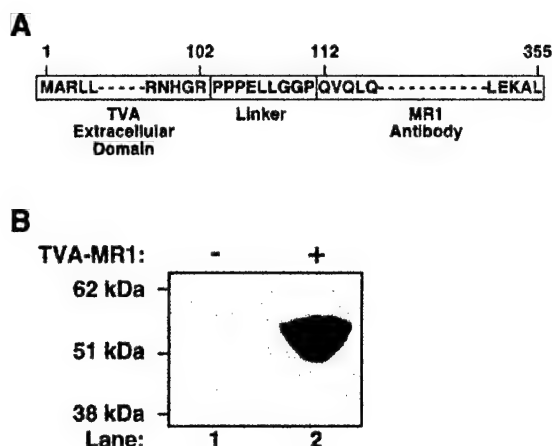


FIG. 1. Production of TVA-MR1. (A) TVA-MR1 was comprised of the extracellular domain of the TVA receptor for ALV-A fused, via a proline-rich linker region, to the N-terminal end of the MR1 single-chain antibody that binds specifically to EGFRvIII. (B) Extracellular supernatants collected from transfected 293 cells that expressed TVA-MR1 (lane 2) or did not express the bridge protein (lane 1) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions. The protein samples were then subjected to immunoblotting with SUA-rIgG (58) and a horseradish peroxidase-conjugated secondary antibody, and the TVA-MR1 protein was detected by enhanced chemiluminescence.

were incubated with increasing amounts of an extracellular supernatant containing TVA-MR1. For control purposes, the parental 293T cells were also incubated with TVA-MR1-containing supernatant. The bridge proteins that were bound to these cells were then detected by flow cytometry after the cells had been incubated with SUA-rIgG, an immunoadhesin composed of the ALV-A surface envelope fused to a rabbit Fc chain (58), and with an FITC-conjugated secondary antibody. TVA-MR1 bound specifically and in a dose-dependent manner to 293T-EGFRvIII cells (Fig. 2A), indicating that this bridge protein can bind simultaneously to both ALV-A SU and to cell surface EGFRvIII.

To obtain direct evidence that TVA-MR1 binds to the EGFRvIII protein, we examined whether the interaction of this bridge protein with 293T-EGFRvIII cells could be blocked by a synthetic peptide that contains the target epitope for the MR1 antibody (29). Indeed, this peptide blocked the binding of TVA-MR1 to these cells (Fig. 2B). By contrast, a control peptide (with the same amino acids scrambled in a different order) did not affect TVA-MR1 binding to these cells (Fig. 2B). To rule out the possibility that the MR1 epitope-containing peptide interfered nonspecifically with ALV-A SU-TVA interactions, this peptide was also tested for its effect on the binding of SUA-rIgG to a stably transfected line of human 293T cells (designated as 293T-TVA<sup>su</sup>) that express a transmembrane form of the TVA receptor. The MR1 epitope-containing peptide did not block the binding of SUA-rIgG to these cells (Fig. 2C), confirming that this peptide specifically blocks the interaction between TVA-MR1 and the EGFRvIII protein.

**TVA-MR1 mediates specific ALV-A entry into 293T-EGFRvIII cells.** To determine whether TVA-MR1 can facilitate ALV-A infection of 293T-EGFRvIII cells, these cells and, for control purposes, 293T cells, were incubated with this bridge protein and then challenged with an ALV-A vector [RCASBP(A)-EGFP], encoding EGFP. The addition of TVA-MR1 led to a significant enhancement of ALV-A entry into

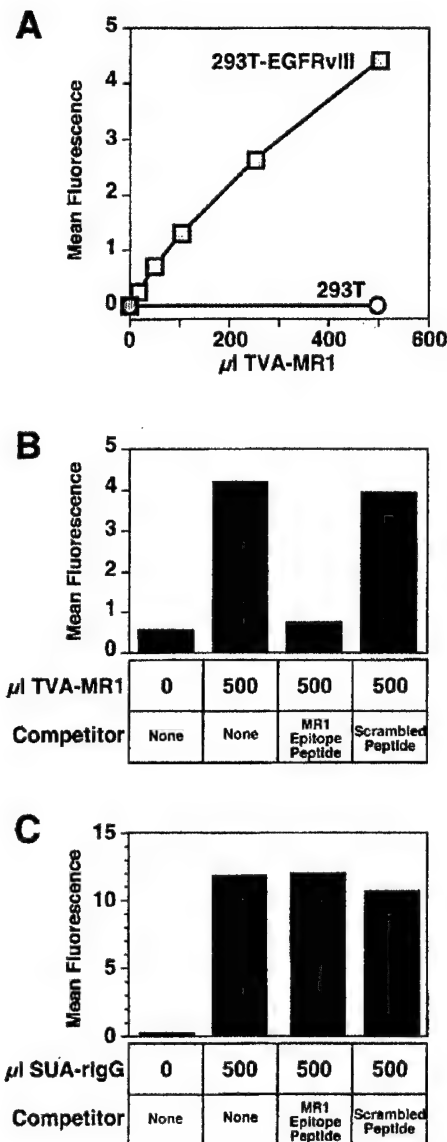


FIG. 2. TVA-MR1 binds specifically to the EGFRvIII protein. (A) 293T cells and 293T-EGFRvIII cells were incubated with extracellular supernatants that either lacked or contained TVA-MR1. The bound TVA-MR1 proteins were then detected by flow cytometry using SUA-rIgG and an FITC-conjugated secondary antibody. (B) Extracellular supernatants containing TVA-MR1 were preincubated with either no peptide (none), the MR1 epitope-containing peptide, or a scrambled version of this peptide. These samples were then incubated with 293T-EGFRvIII cells, and the bound TVA-MR1 proteins were then detected by flow cytometry as shown in panel A. The results obtained were compared with the background levels of fluorescence obtained with cells incubated in the absence of TVA-MR1. (C) 293T-TVA<sup>su</sup> cells were incubated without SUA-rIgG or with SUA-rIgG and with either no peptide (none), the MR1 epitope-containing peptide, or the scrambled peptide. The bound immunoadhesin was then detected by flow cytometry using an FITC-conjugated secondary antibody. Results of a representative experiment are shown (A through C).

293T-EGFRvIII cells (Table 1) (Fig. 3, compare panels B and C). Indeed, TVA-MR1 acted in a dose-dependent manner to increase the susceptibility of these cells to ALV-A infection (data not shown). In these experiments, the level of TVA-MR1-mediated viral entry into 293T-EGFRvIII cells was 8.5%

TABLE 1. Infection of human 293T cell lines by the ALV-A vector RCASBP(A)-EGFP

Cell type	Viral titer <sup>a</sup>	
	- TVA-MR1	+ TVA-MR1
293T	$(5.96 \pm 0.58) \times 10^2$	$(5.36 \pm 0.10) \times 10^2$
293T-EGFRvIII	$(5.94 \pm 0.28) \times 10^2$	$(1.09 \pm 0.01) \times 10^5$
293T-TVA <sup>syn</sup>	$(1.29 \pm 0.02) \times 10^6$	Not done

<sup>a</sup> Titers are expressed per microliter of 100-fold-concentrated virus.

of the level seen with 293T-TVA<sup>syn</sup> cells, and titers of approximately  $10^8$  infectious units/ml of 100-fold-concentrated virus were achieved (Table 1). In contrast, the addition of this bridge protein had no effect upon the susceptibility of the parental 293T cells to viral infection (Table 1) (Fig. 3, compare panels E and F).

To confirm that TVA-MR1 had to be bound to the EGFRvIII protein in order to mediate enhanced levels of viral entry into 293T-EGFRvIII cells, we determined whether this effect could be blocked by the MR1 epitope-containing peptide. The MR1 epitope-containing peptide, but not the control peptide, blocked this bridge protein-dependent viral infection (Fig. 4). Therefore, TVA-MR1 is capable of facilitating targeted ALV-A entry into mammalian cells when bound to the EGFRvIII protein.

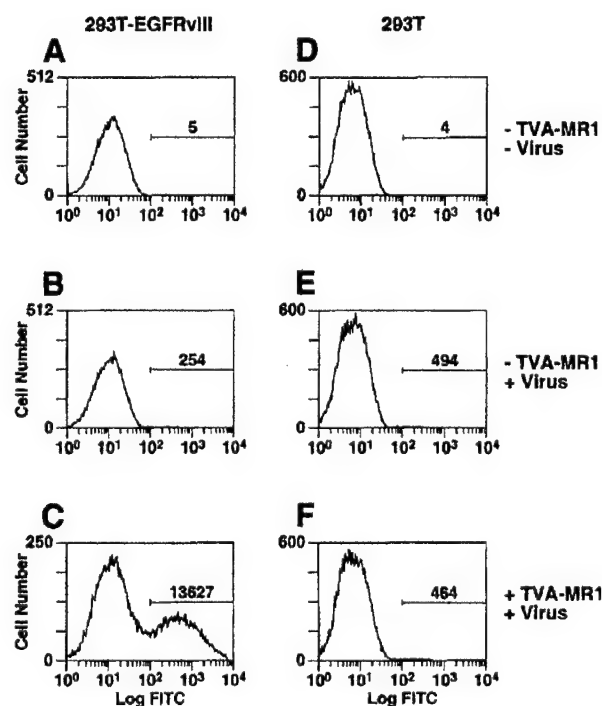


FIG. 3. TVA-MR1 promotes ALV-A infection of 293T-EGFRvIII cells. 293T-EGFRvIII cells (A through C) and 293T cells (D through F) were incubated with extracellular supernatant that contained (+) or lacked (-) TVA-MR1 as indicated. These cells were incubated in the absence or presence of the ALV-A vector RCASBP(A)-EGFP, and then aliquots of each cell type ( $5 \times 10^4$  293T-EGFRvIII cells and  $10^5$  293T cells) were analyzed by flow cytometry.

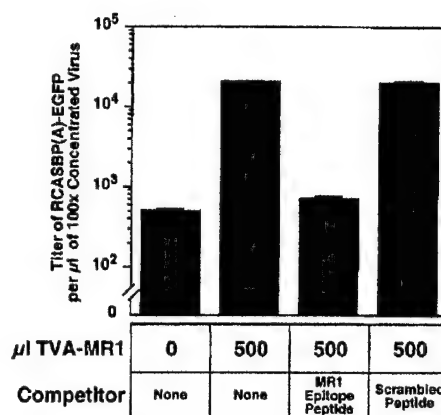


FIG. 4. The TVA-MR1-EGFRvIII interaction is required for targeted viral entry. Extracellular supernatant containing TVA-MR1 was incubated with no peptide (none), the MR1 epitope-containing peptide, or the scrambled peptide before addition to 293T-EGFRvIII cells. The cells were then challenged with RCASBP(A)-EGFP and analyzed by flow cytometry 72 h later. For control purposes, viral infection of 293T-EGFRvIII cells was also assessed in the absence of any TVA-MR1.

## DISCUSSION

In this report we have demonstrated the feasibility of using a soluble retroviral receptor–single-chain antibody bridge protein for targeting retroviral infection to a specific cell type. Cell-binding studies and peptide competition experiments confirmed that TVA-MR1 is a bifunctional reagent that can bind to a cell surface EGFRvIII protein expressed on 293T cells and to ALV-A SU. Furthermore, the binding of TVA-MR1 to the EGFRvIII protein allowed specific viral entry at a level that was approximately 7% (ranging from 1.8 to 14% in separate experiments) of that found in ALV-A infection of 293T-TVA<sup>syn</sup> cells (Table 1 and data not shown). In contrast, TVA-MR1 did not bind to or promote infection of the parental 293T cells, which are known to express cell surface EGFRs (50). Indeed, based upon previous results that demonstrated the unique specificity of the MR1 antibody for EGFRvIII, we fully expected that TVA-MR1 would allow only ALV-A infection of cells expressing mutant, but not wild-type, EGFRs. For example, Lorimer et al. (29) have already shown that this single-chain antibody, engineered in the context of an MR1-toxin fusion protein, directs the potent killing of cells that express 400,000 EGFRvIII molecules (at a concentration of 7 to 10 ng/ml), whereas this recombinant toxin is unable to kill cells expressing 200,000 wild-type EGFR molecules even when added at concentrations as high as 1,000 ng/ml. Taken together, our studies have demonstrated that TVA-MR1 can be an efficient and specific facilitator of viral entry into cells when bound to EGFRvIII.

During these experiments we found that ALV-A is capable of infecting human 293T cells and 293T-EGFRvIII cells at a low “background” level that was 1/2,000 to 1/5,000 of the level seen with 293T-TVA<sup>syn</sup> cells (Table 1 and data not shown). The addition of TVA-MR1 led to an 180-fold to 200-fold increase in the susceptibility of 293T-EGFRvIII cells to ALV-A infection but did not affect the “background” level of infection seen with 293T cells (Table 1). The “background” level of ALV-A infection seen with human 293T cells is similar to that seen with some other human cell lines, e.g., U250 glioma cells, but it is approximately 100-fold higher than that seen with other mammalian cell lines (6). It is important to

understand why certain mammalian cell types are more susceptible to ALV infection than are others, since this information may help us eliminate such infections and thus optimize the use of retroviral receptor-ligand and retroviral receptor-single-chain antibody bridge proteins for viral targeting.

The results presented in this report suggest that it might be possible both in vivo as well as in vitro, to use retroviral receptor-single-chain antibody bridge proteins as tools to deliver retroviral vectors to specific cell types that express cognate cell surface antigens. With regard to in vivo applications, it is not yet clear whether the background level of ALV-A infection that was observed in cultured human 293T cells will represent a significant hurdle for cell type-specific viral targeting: viral targeting studies, performed with transgenic lines of mice that express a transmembrane form of TVA in specific cell types, indicate that the "background" level of ALV-A infection seen with cells that lack this viral receptor is extremely low (16). Furthermore, in considering the potential utility of this system for delivering viral vectors to tumor cells, the use of ALV-based or MLV-based vectors for gene delivery affords another level of specificity since these viruses only establish proviral DNA in dividing cell types (36, 43). Indeed, several groups have already shown that it is possible to use this feature of MLV vectors to deliver genes specifically to tumor cells, even in the absence of a selective Env-targeting system (13, 23). However, since the retroviral vectors used in these studies have a broad host range, there exists the potential for infecting other dividing cell types in addition to the target tumor cells. The use of retroviral receptor-ligand and retroviral receptor-single-chain antibody bridge proteins for viral delivery may lead to more specific viral targeting in vivo. Indeed, it will be interesting to determine the efficiency and specificity of in vivo viral targeting that can be achieved with TVA-MR1 in mouse models of human cancer that employ tumor cells expressing EGFRvIII (21). An added advantage of using retroviral receptor-single-chain antibody fusion proteins is that such bridge proteins might be useful for targeting retroviral vector infection to cells expressing a number of different cell surface factors, including those with no known ligands.

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## Targeting Avian Leukosis Virus Subgroup A Vectors by Using a TVA-VEGF Bridge Protein

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Previously, we have demonstrated that bridge proteins comprised of avian leukosis virus (ALV) receptors fused to epidermal growth factor (EGF) can be used to selectively target retroviral vectors with ALV envelope proteins to cells expressing EGF receptors. To determine whether another type of ligand incorporated into an ALV receptor-containing bridge protein can also function to target retroviral infection, the TVA-VEGF110 bridge protein was generated. TVA-VEGF110 consists of the extracellular domain of the TVA receptor for ALV subgroup A (ALV-A), fused via a proline-rich linker peptide to a 110-amino-acid form of vascular endothelial growth factor (VEGF). This bridge protein bound specifically to its cell surface receptor, VEGFR-2, and efficiently mediated the entry of an ALV-A vector into cells. These studies indicate that ALV receptor-ligand bridge proteins may be generally useful tools for retroviral targeting approaches.

The ability to target viral infection only to specific cell types remains one of the formidable challenges to the use of retroviral vectors for gene therapy. We are developing avian leukosis virus (ALV) receptor-ligand bridge proteins as tools to deliver retroviral vectors to specific cell types. The feasibility of this approach was demonstrated using bridge proteins containing the mature form of human epidermal growth factor (EGF) fused to the extracellular domains of either the TVA receptor or the TVB<sup>S3</sup> receptor for subgroups B and D of ALV. These bridge proteins mediated the highly selective infection of cells that express EGF receptors (3, 23). Recent work by another group has demonstrated adenovirus targeting by using a similar type of bridge protein consisting of the extracellular domain of the coxsackievirus and adenovirus receptor fused to EGF (8).

In the present study, we have tested whether vascular endothelial growth factor (VEGF) can also function as a retroviral targeting ligand when it is introduced into the context of a TVA-containing bridge protein. VEGF is a member of the cysteine-knot growth factor superfamily and is produced as an antiparallel disulfide-linked homodimer with symmetrical receptor-binding sites located at opposite ends of the molecule (27). Alternative splicing of a common primary mRNA transcript generates differently sized ligand isoforms: VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206 (27). The murine VEGF110 form that was used in this study consists of the N-terminal 110 amino acids of VEGF165, with the C-terminal heparin-binding domain (7) removed to reduce non-specific binding of the bridge protein to cell surfaces.

Three different types of VEGF receptors have been identi-

fied: VEGFR-1, VEGFR-2, and VEGFR-3 (27). VEGF receptors are selectively expressed on the surfaces of endothelial cells (27). In addition to these three receptors, the NRP-1 protein that is a receptor for collapsins and semaphorins is also a receptor for VEGF165 (27). Compared to VEGF165, VEGF110 has the same binding affinity for VEGFR-2, a lower affinity for VEGFR-1, and does not bind to NRP-1 (15, 25).

VEGF is important to test as a potential ligand for retroviral targeting because it binds to receptors that are expressed on tumor vasculature. Solid tumors require the presence of a network of blood vessels to obtain oxygen and nutrients for their growth (10). To induce formation of new blood vessels, a process termed angiogenesis, tumors express a variety of growth factors, one of which is VEGF (5, 9, 12, 13, 14, 18, 22, 26). VEGF is known to specifically induce growth and migration of endothelial cells as well as to cause permeability of blood vessels, and inhibitors of VEGF signaling retard tumor growth in mice (11, 16, 19–21).

The TVA-VEGF110 protein consists of the extracellular domain of TVA fused via a proline-rich hinge region to murine VEGF110 (Fig. 1A). Additional bridge proteins were also generated, consisting of the extracellular domain of TVB<sup>S3</sup> fused via the same hinge region to either VEGF110 or the EGF-like region of human heregulin- $\beta$ 1 (her $\beta$ 1), respectively (Fig. 1A). Production of each bridge protein in the extracellular supernatant of transiently transfected human 293 cells was confirmed after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with a subgroup A- or a subgroup B-specific surface (SU)-immunoglobulin fusion protein (SU-Ig) to detect TVA- and TVB-containing bridge proteins, respectively, as described previously (3, 23). Under non-reducing conditions, TVA-VEGF110 migrated as an 84- to 115-kDa protein species (Fig. 1B), consistent with it being a disulfide-linked dimer like VEGF (see Fig. 1A legend for a description of the expected molecular mass of this protein).

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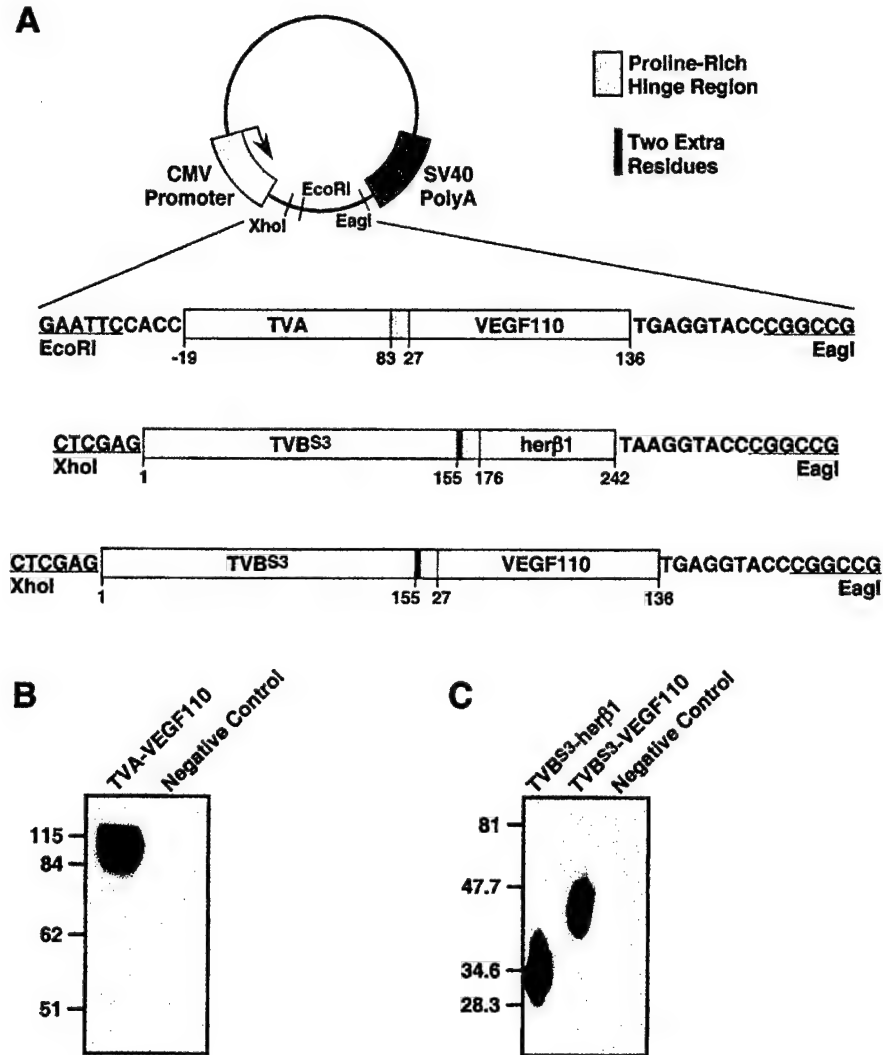


FIG. 1. Construction and expression of retroviral receptor-ligand bridge proteins. (A) Recombinant genes encoding each bridge protein were generated by PCR-based methods and introduced into the pCI-plasmid expression vector (Promega) as shown. The numbering schemes for the amino acid residues of TVA, TVBS<sup>3</sup>, and heregulinβ1 were taken from references 2 and 6 and GenBank accession number B43273, respectively. The VEGF110 residues are described under GenBank accession number A44881. The positions of a proline-rich hinge region (PPPELLGGP) and of a 2-amino-acid insertion (His-Gly) that resulted during the construction of the TVBS<sup>3</sup>-containing bridge proteins are indicated. The TVA-VEGF110 monomer was expected to have a molecular mass ranging from 33 to 52 kDa because the primary amino acid sequence predicts a 22.4-kDa protein but the extracellular domain of TVA is subjected to extensive posttranslational modifications which add an additional 21 to 30 kDa to its apparent molecular mass (1, 2). Monomeric forms of TVBS<sup>3</sup>-VEGF110 and TVBS<sup>3</sup>-herβ1 were expected to have molecular masses of 37 and 33 kDa, respectively, based on their primary amino acid sequences (28 and 24 kDa, respectively) and the presence of three putative N-linked glycosylation sites in each protein. (B and C) Production of bridge proteins. Forty-five-microliter aliquots of extracellular supernatant taken from transfected human 293 cells that expressed the bridge proteins, or from nontransfected cells (negative controls), were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under either nonreducing (B) or reducing (C) conditions. The proteins were then transferred to a nitrocellulose membrane and were probed with subgroup A-specific (panel B) or subgroup B-specific (panel C) SU-rIg fusion proteins and then with a horseradish peroxidase-conjugated secondary antibody, as described previously (3, 23). The bridge proteins were then detected by enhanced chemiluminescence.

Under reducing conditions, the TVBS<sup>3</sup>-containing bridge proteins migrated at positions that were consistent with their expected monomeric molecular masses (Fig. 1C).

Flow cytometry was performed to analyze the binding of TVA-VEGF110 to porcine aortic endothelial (PAE) cells that express few or no VEGF receptors (17, 25, 28) or to transduced

PAE cells expressing mouse VEGFR-2 (PAE-VEGFR-2 cells). PAE-VEGFR-2 cells were generated by transduction of PAE cells with a VSV-G pseudotyped murine leukemia virus (MLV) vector encoding VEGFR-2 [MLV(VSV-G)-VEGFR-2]. The pseudotyped virus was produced from human 293T cells plated at 60% confluence on 100-mm tissue culture plates.

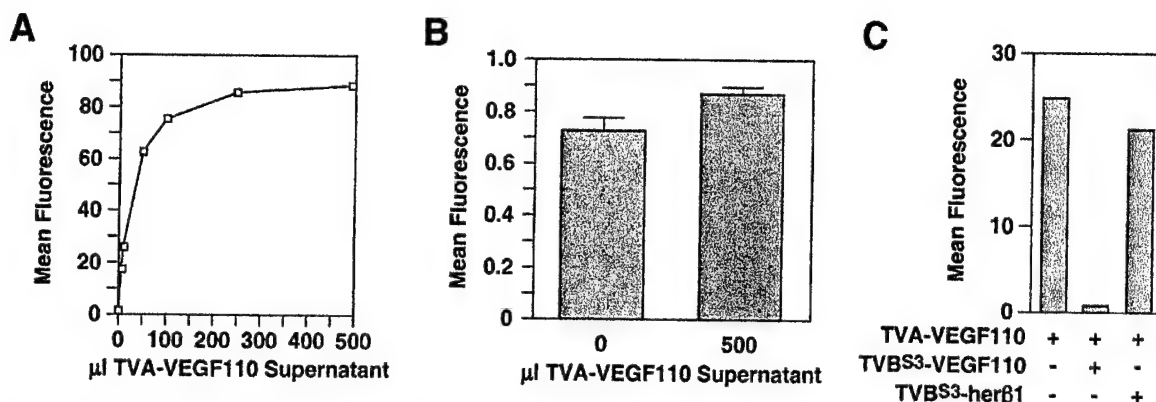


FIG. 2. TVA-VEGF110 binds specifically to cells that express VEGFR-2. PAE-VEGFR-2 cells (A) and PAE cells (B) were incubated with increasing amounts of a TVA-VEGF110-containing extracellular supernatant. (C) Prior to incubation with TVA-VEGF110, PAE-VEGFR-2 cells were incubated with extracellular supernatant that contained either TVBS<sup>3</sup>-VEGF110, TVBS<sup>3</sup>-herβ1, or no TVB-ligand bridge protein. Following these treatments the TVA-VEGF110 protein that was bound to the cells was detected by flow cytometric analysis using a subgroup A-specific SU-rIg fusion protein and an FITC-conjugated secondary antibody as described previously (23). These experiments were performed three times with similar results, and results of a representative example are shown.

These cells were transiently transfected with 5 μg of pMD.G plasmid encoding the VSV-G protein, 15 μg of pMMD.gagpol plasmid encoding MLV Gag and Gag-Pol structural proteins (24), and 15 μg of pSFG.Flk1 plasmid encoding VEGFR-2 (unpublished data). A 30%-confluent well of a six-well plate of PAE cells was then incubated with 1 ml of MLV(VSV-G)-VEGFR-2 in the presence of 8 μg of Polybrene per ml. The resultant VEGFR-2-expressing cells were then isolated by flow cytometric sorting after incubation with supernatants containing TVA-VEGF110 and SUA-rIgG, and then with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (data not shown).

To assay for specific binding of TVA-VEGF110 to VEGF receptor-expressing cells,  $3.5 \times 10^5$  PAE-VEGFR-2 cells and the same number of control PAE cells were incubated for 1 h at 4°C with different amounts of a TVA-VEGF110-containing supernatant that was supplemented with a control 293 cell-conditioned medium to a total volume of 500 μl. The cells were then washed with ice-cold phosphate-buffered saline (PBS) (containing 2% fetal bovine serum) and then incubated with SUA-rIgG and an FITC-conjugated secondary antibody and subjected to flow cytometric analysis as described before (23). Because the bound TVA-VEGF110 protein was detected with a soluble SU reagent, these studies also established whether the bridge protein can bind simultaneously to cell surface VEGF receptors and to ALV subgroup A (ALV-A) SU. Indeed, TVA-VEGF110 bound in a dose-dependent manner to PAE-VEGFR-2 cells (Fig. 2A) but reproducibly bound only weakly to PAE cells (Fig. 2B), perhaps indicating that these cells do in fact express a small number of VEGF receptor(s). These binding studies supported the idea that TVA-VEGF110 can serve as a bridge between cell surface VEGFR-2 and ALV-A SU.

To formally show that TVA-VEGF110 binds to VEGFR-2, competition binding experiments were performed in the presence of heterologous bridge proteins that either contained the same (TVBS<sup>3</sup>-VEGF110) or different (TVBS<sup>3</sup>-herβ1) ligand moieties (Fig. 1). The competition binding experiments were

performed by incubating  $3.5 \times 10^5$  PAE-VEGFR-2 cells at 4°C for 1 h with 490 μl of extracellular supernatants that contained equivalent amounts (as judged by quantitative chemiluminescence using a Bio-Rad FluorS instrument) of either TVBS<sup>3</sup>-herβ1 or TVBS<sup>3</sup>-VEGF110. A 10-μl aliquot of a TVA-VEGF110-containing supernatant was then added and the cells were incubated at 4°C for an additional hour. The cells were then washed in PBS and analyzed by flow cytometry using SUA-rIgG and the FITC-conjugated antibody as before. TVA-VEGF110 binding was blocked by preincubation with TVBS<sup>3</sup>-VEGF110 but not with TVBS<sup>3</sup>-herβ1 (Fig. 2C). These data confirm that TVA-VEGF110 binds specifically to VEGFR-2 expressed at the surface of PAE-VEGFR-2 cells.

To determine whether TVA-VEGF110 can mediate ALV-A entry when bound to VEGFR-2, approximately  $10^5$  PAE-VEGFR-2 cells were incubated for 1 h at 4°C with increasing amounts of a TVA-VEGF110-containing supernatant that was made up to a total volume of 500 μl with control supernatant taken from nontransfected human 293 cells. The cells were then washed with ice-cold medium and incubated with 500 μl of ice-cold medium containing 5 μl of a 100-fold concentrated stock of an ALV-A vector RCASBP(A)-EGFP encoding the enhanced green fluorescent protein, which was prepared as described elsewhere (24).

Approximately 72 h after viral challenge, the cells were washed with PBS and removed from plates with Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS containing 1 mM EDTA and 7 μM propidium iodide. The infected cells were then identified by flow cytometry and dead cells that had taken up propidium iodide were excluded from the analysis by electronic gating. These studies showed that TVA-VEGF110 rendered PAE-VEGFR-2 cells susceptible to ALV-A infection in a dose-dependent manner (Fig. 3A).

To determine the efficiency and specificity of TVA-VEGF110-dependent infection, parental PAE cells and PAE-TVA<sup>syn</sup> cells which express a transmembrane form of TVA were also challenged with the ALV-A vector. PAE-TVA<sup>syn</sup> cells were generated by transducing PAE cells with an MLV

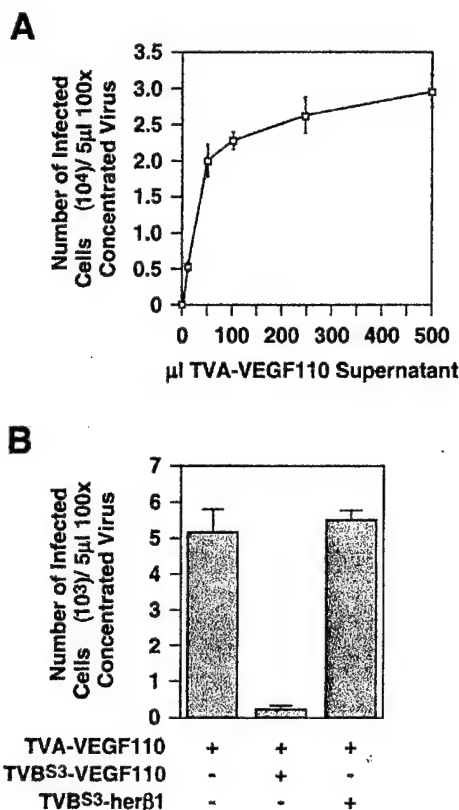


FIG. 3. TVA-VEGF110 mediates ALV-A infection when bound to VEGFR-2. (A) PAE-VEGFR-2 cells were incubated with increasing amounts of extracellular supernatant containing TVA-VEGF110 and then challenged with 5  $\mu$ l of a 100-fold concentrated stock of RCASBP(A)-EGFP. The total number of infected cells obtained was then calculated by flow cytometry as described previously (24). The average data obtained from an experiment that was performed in triplicate are shown with standard deviations indicated with error bars. (B) PAE-VEGFR-2 cells were incubated with equivalent amounts of TVBS<sup>3</sup>-VEGF110 or TVBS<sup>3</sup>-her $\beta$ 1 or with no TVB-ligand bridge protein, prior to the addition of 10  $\mu$ l of TVA-VEGF110. The cells were then challenged with RCASBP(A)-EGFP as in panel A and analyzed by flow cytometry. The results of an experiment performed in triplicate are shown with standard deviations of the data indicated with error bars.

vector encoding a synthetic transmembrane form of the TVA receptor (2). The RCASBP(A)-EGFP titer that was obtained with these cells was approximately  $7.5 \times 10^7$  infectious units/ml of 100-fold-concentrated virus (defined as 100%, Table 1). Strikingly, TVA-VEGF110-mediated infection of PAE-VEGFR-2 cells was only 11.3-fold less than the level seen with the control TVA-expressing cells (Table 1). Furthermore, in the absence of TVA-VEGF110, only low levels of ALV infection were observed (Table 1), consistent with the previously published "background" levels of ALV infection seen in various mammalian cell types (4). The addition of TVA-VEGF110 to the control PAE cells did lead to a slight enhancement of viral entry (Table 1), a result which again indicates that these cells may express a low number of VEGF receptors (Fig. 2B).

To confirm that the TVA-VEGF110-VEGFR-2 interaction is necessary for the enhanced viral entry seen with PAE-

TABLE 1. TVA-VEGF110-dependent infection of PAE-VEGFR-2 cells

Cell type	Efficiency of ALV-A infection (%) <sup>a</sup>	
	Without TVA-VEGF110	With TVA-VEGF110
PAE-TVA <sup>syn</sup>	100	Not done
PAE	0.01	0.09
PAE-VEGFR-2	0.02	8.72

<sup>a</sup> RCASBP(A)-EGFP titer relative to that in PAE-TVA<sup>syn</sup> cells ( $7.5 \times 10^7$  infectious units/ml, defined as 100%).

VEGFR-2 cells, we attempted to block this infection by incubating these cells at 4°C for 30 min with equivalent amounts of TVBS<sup>3</sup>-VEGF110 or TVBS<sup>3</sup>-her $\beta$ 1 prior to adding the TVA-containing bridge protein as before (Fig. 2C). The cells were then challenged with RCASBP(A)-EGFP and analyzed by flow cytometry as described above. TVA-VEGF110-dependent infection of PAE-VEGFR-2 cells was inhibited by TVBS<sup>3</sup>-VEGF110 but not by TVBS<sup>3</sup>-her $\beta$ 1, thereby confirming that the VEGF110-VEGFR-2 interaction is essential for bridge protein-enhanced viral entry (Fig. 3B).

Taken together, the studies presented in this report clearly demonstrate that targeted ALV-A vector entry can be achieved through the TVA-VEGF110-VEGFR-2 interaction. TVA-VEGF110 bound specifically to cells that express VEGFR-2 and mediated efficient infection of these cells by an ALV-A vector. This system for viral targeting represents an attractive model for the development of retroviral vectors that can be targeted to tumor vasculature. Furthermore, these findings, coupled with the demonstration of retroviral targeting via bridge proteins containing the EGF ligand (3, 23) or a single-chain antibody raised against a tumor-specific form of the EGF receptor (24), indicate that ALV receptor-containing bridge proteins may be generally useful reagents for cell-type-specific retroviral targeting.

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# Appendix 4

## **Targeting Retroviral Vector Infection to Cells that Express Heregulin Receptors using a TVA-Heregulin Bridge Protein**

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Running Title: Retroviral targeting via heregulin receptors

## ABSTRACT

Previously we have shown that it is possible to target retroviral vectors to cells using avian sarcoma and leukosis virus (ASLV) receptor-ligand and receptor-single chain antibody bridge proteins (now designated as GATEs for Guided Adaptors for Targeted Entry). In this report we were interested in determining whether or not this approach can be used to deliver retroviral vectors specifically to cells that express heregulin receptors. Heregulin receptors are attractive targets for retroviral vector-based gene delivery protocols since they are often overexpressed on the surfaces of cancer cells. To explore this possibility, the TVA-her $\beta$ 1 protein was generated, consisting of the extracellular domain of the TVA receptor for ASLV-A fused to the EGF-like region of heregulin  $\beta$ 1. TVA-her $\beta$ 1 bound specifically to cells that express heregulin receptors, rendering them susceptible to efficient and specific infection by subgroup A ASLV vectors. In addition, these activities of TVA-her $\beta$ 1 were abrogated specifically in the presence of another bridge protein that contained the same ligand domain. These data confirm that the GATE protein TVA-her $\beta$ 1 and mediate targeted retroviral infection via cell surface heregulin receptors.

Key words: retrovirus, targeting, ASLV, bridge proteins, heregulin, GATE

## INTRODUCTION

The ability to target viral vectors carrying therapeutic or suicide genes to specific cell types would be of great use in gene therapy of cancers. Retroviruses such as avian sarcoma and leukosis viruses (ASLVs) or murine leukemia viruses (MLVs) are advantageous in this regard since they infect, and predominantly establish proviral DNA in, actively dividing cells. The most common approach toward retargeting of viral vectors has been to modify retroviral envelope (Env) proteins to contain a ligand or a single chain antibody (scAb) that bind a particular cell surface marker (Chadwick et al., 1999, Hatzioannou et al., 1999, Jiang et al., 1998; Jiang et al., 1999, Kasahara, Dozy, and Kan, 1994, Konishi et al., 1998, Martin et al., 1999, Nilson et al., 1996, Peng et al., 1999, and reviewed in Verma and Somia, 1997, Cosset and Russell, 1996). Alternatively, retroviral targeting has been attempted using molecular bridges consisting of a retroviral Env-specific antibody that is crosslinked to either a ligand or to another antibody that binds a specific cell surface marker (Goud, Legrain, and Buttin, 1988, Etienne-Julan et al., 1992, Roux, Jeanteur, and Piechaczyk, 1989). However, difficulties with production of viral particles containing modified Env proteins and a block to virus cell membrane fusion have limited the progress of these methods (Zhao et al., 1999, Verma and Somia, 1997, Cosset and Russell, 1996).

Recently, we and our collaborators were able to direct ALV- and MLV-based retroviral vectors to specific cell types using ASLV receptor-ligand, and ASLV receptor-single chain antibody, bridge proteins (Boerger, Snitkovsky, and Young, 1999, Snitkovsky et

al., 2000; Snitkovsky et al., 2001; Snitkovsky and Young, 1998). These bridge proteins contain the extracellular domains of either the TVA or TVB receptors for subgroups A and B ASLV (ASLV-A and ASLV-B), respectively. The bridge proteins that have been tested to date have the ASLV receptor domains fused in-frame with either epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), or a single-chain antibody (MR1) that binds to a tumor-specific form of the EGF receptor. These bridge proteins efficiently targeted the entry of retroviral vectors to cells that express the cognate cellular receptors (Snitkovsky and Young 1998, Boerger, Snitkovsky, and Young 1999, Snitkovsky et al. 2000, Snitkovsky et al. 2001).

In this report, we have tested whether this approach for retroviral targeting can be used to direct viral infection toward cells that express heregulin receptors. The heregulin receptors erbB2, erbB3 and erbB4 belong to a family of class I tyrosine kinases that includes EGFR (also designated as erbB1) (Carraway and Cantley, 1994, Plowman et al., 1993; Plowman et al., 1990). Homodimers of erbB3 and erbB4 can serve as heregulin receptors (Tzahar et al., 1994) and erbB2 binds heregulins only when it forms a heterodimer with erbB3 or erbB4 (Peles et al., 1993, Sliwkowski et al., 1994).

Heregulin receptors are relevant cell surface targets for a retroviral targeting approach since ErbB2 is often overexpressed in various malignancies, including breast and ovarian carcinomas (Slamon et al., 1989), and clinical studies have correlated overexpression of erbB2 in female breast cancers with resistance to hormone therapy, recurrence after surgery and radiation, and poor survival (Giovannella et al., 1991, Ross and Fletcher,

1999, Slamon et al., 1987; Slamon et al., 1989). Also, heterodimers of erbB2 and erbB3 are expressed in a variety of tumor derived cells where they serve as high affinity binding sites for heregulins (Sliwkowski et al., 1994, Tzahar et al., 1994).

Heregulin  $\beta 1$  is one of the multiple heregulin isoforms resulting from alternative splicing of a single gene (Holmes et al., 1992, Wen et al., 1992). The EGF-like domains of heregulins are sufficient for binding to heregulin receptors and for mediating mitogenic signaling (Holmes et al., 1992, Carraway et al., 1995). Among all heregulin isoforms, the EGF-like domain of heregulin  $\beta 1$  has the highest affinity for these heterodimeric receptors (Tzahar et al., 1994). Here we show that a TVA-heregulin  $\beta 1$  bridge protein (TVA-her $\beta 1$ ) that contains this EGF-like domain specifically directs ASLV-A vector infection to cells expressing the ErbB2 and ErbB3 heregulin receptors.

## RESULTS

### **TVA-her $\beta$ 1 binds specifically to N234 cells**

A synthetic gene encoding TVA-her $\beta$ 1 was constructed as described in Materials and Methods. TVA-her $\beta$ 1 consists of the extracellular domain of TVA fused via a proline-rich linker region to the N-terminus of the EGF-like domain of heregulin  $\beta$ 1. TVA-her $\beta$ 1 was produced as a secreted protein in the extracellular supernatant of transiently-transfected 293 cells and its expression was confirmed by immunoblotting. TVA-her $\beta$ 1 migrated as a heterogeneous protein species ranging from 32 to 43 kDa (Fig. 1A), most probably because its TVA domain is extensively modified post-translationally (Bates, Young, and Varmus, 1993).

TVA-her $\beta$ 1 was tested for its cell-specific binding properties using NIH 3T3 cells that lack heregulin receptors (Carraway et al., 1995), and transfected NIH 3T3 cells (N234 cells) that express the erbB2/erbB3 heregulin receptors (Carraway et al., 1995). The cells were incubated with extracellular supernatant that either lacked or contained TVA-her $\beta$ 1 and the bound bridge protein was detected using a previously described flow cytometry-based assay that employs SUA-rIgG, a soluble subgroup A-ASLV SU-immunoglobulin fusion protein (Snitkovsky et al. 1998, Snitkovsky et al. 2000, Snitkovsky et al. 2001). TVA-her $\beta$ 1 bound to N234 cells in a concentration dependent manner (Fig. 1B), whereas it did not bind at all to NIH 3T3 cells even when the maximal amount of bridge protein was added (Fig. 1C). These studies clearly show that TVA-her $\beta$ 1 binds specifically to cells that express heregulin receptors. In addition, since the cell surface-associated bridge

protein was detected by binding to SUA-rIgG, these studies confirm that the bound protein is capable of interacting directly with subgroup A ASLV Env.

### **TVA-her $\beta$ 1 mediates efficient and specific viral entry into N234 cells**

To determine whether TVA-her $\beta$ 1 can mediate infection by ASLV-A vectors into cells that express heregulin receptors, N234 cells were incubated with increasing amounts of this bridge protein prior to challenge with RCASBP(A)-EGFP, a subgroup A ASLV vector encoding the enhanced green fluorescent protein (EGFP) (Snitkovsky et al. 2000, Snitkovsky et al. 2001). Infected cells were identified by flow cytometry to detect EGFP expression. These studies demonstrated that TVA-her $\beta$ 1 can mediate ALV-A infection of N234 cells in a concentration-dependent manner (Fig. 2). To estimate the relative efficiency of TVA-her $\beta$ 1-mediated infection of N234 cells as compared with that mediated by the transmembrane TVA receptor, NIH 3T3-TVA<sup>syn</sup> cells were also challenged with virus. These cells were derived from NIH 3T3 cells by transduction with an MLV vector that encodes an epitope-tagged form of the transmembrane TVA receptor (Snitkovsky 2000, Snitkovsky 2001). These studies showed that TVA-her $\beta$ 1-mediated infection was really quite efficient since it led to viral transduction at a level that was approximately 5% of that seen with cells expressing the transmembrane receptor (Table 1). By contrast to the results obtained with N234 cells, TVA-her $\beta$ 1 failed to render the parental NIH 3T3 cells susceptible to viral infection (Table 1).

## **TVA-her $\beta$ 1-mediated viral entry requires its binding to cell surface heregulin receptors**

To confirm that TVA-her $\beta$ 1-mediated viral entry requires its binding to heregulin receptors expressed at the surface of N234 cells, we attempted to compete these activities of the bridge protein with two other bridge proteins, TVB<sup>S3</sup>-her $\beta$ 1 and TVB<sup>S3</sup>-VEGF110 (Snitkovsky 2001). These two bridge proteins consist of the extracellular domain of the TVB<sup>S3</sup> receptor for subgroups B and D ASLV fused via a proline-rich linker region to either the EGF-like region of heregulin $\beta$ 1 or to a 110-amino acid form of VEGF (Snitkovsky et al. 2001). Expression of each of these two proteins in the extracellular supernatant of transiently-transfected human 293 cells was quantified previously (Snitkovsky 2001). TVB<sup>S3</sup>-her $\beta$ 1 blocked the binding of TVA-her $\beta$ 1 to N234 cells (Fig. 3A) and severely reduced the level of TVA-her $\beta$ 1-mediated viral infection that was observed with these cells (Fig. 3B). By contrast, preincubating N234 cells with TVB<sup>S3</sup>-VEGF110 had no effect upon TVA-her $\beta$ 1-binding (Fig. 3A) or viral entry (Fig. 3B). Taken together, these studies confirm that TVA-her $\beta$ 1 binds to heregulin receptors expressed at the surface of N234 cells and demonstrates that this interaction is required for the bridge protein-mediated viral entry.

## DISCUSSION

In this report we have demonstrated that it is possible to target retroviral infection specifically toward cells that express heregulin receptors using a TVA-heregulin bridge protein (TVA-her $\beta$ 1). Specifically we showed that this bridge protein binds to transfected NIH 3T3 cells that express heregulin receptors (N234 cells) and that its binding was specifically abrogated by a heterologous bridge protein (TVB<sup>S3</sup>-her $\beta$ 1) that binds the same receptor. Furthermore, TVA-her $\beta$ 1 rendered N234 cells susceptible to infection by a subgroup A ASLV vector in a concentration-dependent manner, and this activity was also inhibited by prior incubation of the cells with TVB<sup>S3</sup>-her $\beta$ 1. The ability to target retroviral vectors via cell surface heregulin receptors is an important first step for the development of future gene therapy efforts that are aimed at eliminating tumors that overexpress this type of receptor.

The efficiency of TVA-her $\beta$ 1-mediated viral entry was approximately 5% of that level seen with modified NIH 3T3 cells that express the transmembrane TVA receptor. This level of targeted viral entry compares favorably with the previous data obtained when similar experiments were performed with other bridge proteins that target retroviral infection through wild-type EGF receptors (6.6%; Snitkovsky 1998), through VEGFR-2 (8.72%; Snitkovsky 2001) and through a tumor-specific form of the EGF receptor, EGFRvIII (8.4%; Snitkovsky 2000). Therefore, this approach seem to give consistent results with several different receptor-ligand pairs.

ASLV receptor-ligand. and ASLV receptor-single chain antibody, bridge proteins direct retroviral infection toward specific cell surface receptors. Therefore, now designate these proteins collectively as GATEs (Guided Aaptors for Targeted Entry). The use of GATE proteins is not limited to targeting only ASLV or MLV vectors since it can, in principle, be applied to any virus vector system that can incorporate ASLV Env proteins.

Furthermore, recent studies have demonstrated that a similar approach results in adenovirus vector targeting using coxsackie B virus and adenovirus receptor (CAR)-EGF and CAR-Fc bridge proteins (Dmitriev et al., 2000, Ebbinghaus et al., 2001). The versatility of bridge proteins in targeting different classes of viruses to cell types expressing different kinds of receptors may in the future permit successful gene therapy in humans.

## MATERIALS AND METHODS

**Cell lines.** NIH3T3 cells were grown in DMEM containing 5% FBS, L-glutamine, and penicillin/streptomycin (DMEM). N234 cells were derived from NIH3T3 cells by transfection of ErbB2 and ErbB3 (a generous gift of K. Carraway), and were grown in DMEM containing 800 µg/ml Geneticin (G418) (GIBCO/BRL). NIH3T3 cells expressing TVA<sup>syn</sup> (NIH3T3-TVA<sup>syn</sup>) were generated by infecting NIH3T3 cells with an MLV vector encoding TVA<sup>syn</sup>, pseudotyped with the VSV-G protein as described previously (Snitkovsky et al., 2000, Snitkovsky et al. 2001).

**Generation of the TVA-heregulin β1 fusion protein.** A synthetic gene encoding a proline-rich linker (Snitkovsky and Young 1998) fused to the N-terminus of the 68 amino acids of the EGF-like domain of heregulin β1 (Holmes et al., 1992) was obtained by a PCR amplification method (Soderlind, Vergeles, and Borrebaeck, 1995) using six overlapping complementary oligonucleotide primers that span the sequence of the EGF-like domain of heregulin β1. The primers used (arranged in order from the 5'-end of the synthetic gene) were the sense primer #1 (5'-  
gcgcggccgcccaccacctaactcctagggggaccggggaccagccacctgtgaaatg-3') with the EagI cloning site, and the codons encoding the proline-rich hinge region underlined; the antisense primer #2 (5'-  
ctcgccgccgttcacgcagaaagtttctccttctccgcacatttcacaaggtggctgggtccccgg-3' ); the sense

primer #3 (5'-

ctttctgctgaacggcgaggagtgcttcattggttaaagacctgtccaacccctcgcgctacttgcaagtcccaaatgagttc

accggcgatcgctgccaaaactacgtaatggcct-3'); the antisense primer #4 (5'-

ggcagcgatcgccggtgaactcatttgggcacttgacaaagtagcgcgaggggttgacagg-3'); the sense primer

#5 (5'-gagttcaccggcgatcgctgccaaaactacgtaatggcctccttctacaagcacctggggatcg-3'); the

antisense primer #6 (5'-

gcgcggccgggtaccttactcctccgctccataaattcgatccccaggtgctttagaagg-3') with the EagI

cloning site underlined. In brief, 30 pmols of primers #1 and #6 were mixed with 0.3

pmols of primers #2, #3, #4, and #5, in a standard PCR amplification reaction. The

resultant 259 base pair-long DNA fragment was digested with EagI restriction enzyme,

and was used to replace the corresponding proline-rich and ligand regions of TVA-EGF

(Snitkovsky and Young, 1998) generating a plasmid expression vector encoding TVA-

herβ1.

The TVA-herβ1 protein, as well as the previously described TVB<sup>S3</sup>-herβ1 and TVB<sup>S3</sup>-

VEGF110 proteins (Snitkovsky et al. 2001), were expressed in the extracellular

supernatants of transiently-transfected human 293 cells as previously described

(Snitkovsky and Young 1998). Expression of TVA-herβ1 was confirmed by subjecting

45 μl of the extracellular supernatant to electrophoresis on a 10% polyacrylamide gel

containing SDS. The protein was then transferred to a nitrocellulose membrane and was

detected by immunoblotting using an ALV-A surface (SU) Env-rabbit IgG fusion protein

(SUA-rIgG) as described previously (Snitkovsky 2000).

**TVA-herβ1 binding studies.** Approximately  $3.5 \times 10^5$  cells were detached from tissue culture plates with  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS containing 1mM EDTA and placed on ice. N234 cells were incubated at 4°C for 1 h with increasing amounts (up to 500 μl) of the extracellular supernatant taken from TVA-herβ1 expressing cells with each sample made up to a total volume of 500 μl with a control supernatant taken from non-transfected human 293 cells. NIH 3T3 cells were incubated with 500 μl of the control or TVA-herβ1-containing supernatants. The cells were then washed and subjected to a flow cytometry assay using SUA-rIgG to detect the bound bridge protein as described previously (Snitkovsky 2000). The competition binding studies were performed in the same way except that the N234 cells were preincubated for 1 h at 4°C with 490 μl of a control supernatant or with extracellular supernatants that contained TVB<sup>S3</sup>-herβ1 or TVB<sup>S3</sup>-VEGF110 before the addition of a 10 μl sample of supernatant taken from TVA-herβ1-expressing cells.

**Viral Infection.** All of the experiments described in this section were performed at least three times each in triplicate. Approximately  $10^5$  N234, NIH 3T3, and NIH3T3-TVA<sup>syn</sup> cells were incubated for 1 h at 4°C with 500 μl of a control supernatant taken from non-transfected human 293 cells. The NIH 3T3 cells were also incubated for under the same conditions with 500 μl of a supernatant taken from TVA-herβ1-expressing cells. N234 cells were also incubated under the same conditions with increasing amounts of the TVA-herβ1-containing supernatant and in each case the samples were made up to a total volume of 500 μl with the control supernatant. The cells were then washed, challenged

with RCASBP(A)-EGFP, and the infected cells were identified by flow cytometry as described previously (Snitkovsky 2000). The competition experiments were performed in the same way except that N234 cells were preincubated at 4°C for 30 min with 490 µl of the control supernatant or instead with 490 µl of the supernatants containing TVB<sup>S3</sup>-herβ1 or TVB<sup>S3</sup>-VEGF110 before the addition of 10 µl of the TVA-herβ1-containing supernatant.

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## FIGURE LEGENDS

Figure 1. TVA-her $\beta$ 1 binds specifically to N234 cells that express heregulin receptors. A. Expression of TVA-her $\beta$ 1. An aliquot of extracellular supernatant containing TVA-her $\beta$ 1 was subjected to SDS-PAGE under non-reducing conditions and then to immunoblotting using a subgroup A-ASLV SU-immunoglobulin fusion protein (SUA-rIgG) and a horseradish peroxidase conjugated secondary antibody as described previously (Snitkovsky and Young 1998). Molecular weight markers are shown in kD. B. TVA-her $\beta$ 1 binds to N234 cells. The N234 cells were incubated with supernatants that contained an increasing amount of TVA-her $\beta$ 1 and the bound bridge protein was then detected by flow cytometry using SUA-rIgG and a FITC-conjugated secondary antibody as described previously (Snitkovsky and Young 1998). C. TVA-her $\beta$ 1 does not bind to NIH 3T3 cells. The NIH 3T3 cells were incubated with 500  $\mu$ l extracellular supernatants that either lacked (-) or contained (+) TVA-her $\beta$ 1 and the bound bridge protein was detected by flow cytometry as described in panel B.

Figure 2. TVA-her $\beta$ 1 mediates infection by a subgroup A ASLV vector in a dose-dependent manner. N234 cells were incubated with extracellular supernatant containing increasing amounts of TVA-her $\beta$ 1. The cells were then washed and were challenged with a 5  $\mu$ l aliquot of a (100 x concentrated) stock of the RCASBP(A)-EGFP viral vector (Snitkovsky et al. 2000, Snitkovsky et al. 2001). The number of virus-transduced cells was then determined by flow cytometry.

Figure 3. TVA-herβ1 mediates viral entry into N234 cells by binding to heregulin receptors. A. TVA-herβ1 binding to N234 cells is blocked in the presence of a heterologous bridge protein (TVB<sup>S3</sup>-herβ1) that also contains the heregulin β1 domain. N234 cells were incubated with extracellular supernatant that either contained (+) or lacked (-) TVB<sup>S3</sup>-herβ1 or TVB<sup>S3</sup>-VEGF110 prior to incubation with TVA-herβ1. The bound TVA-herβ1 protein was detected by flow cytometry as described in panel B, Fig. 1. B. TVB<sup>S3</sup>-herβ1 blocks TVA-herβ1-dependent viral entry into N234 cells. N234 cells were incubated with bridge proteins as described in panel A and after washing they were challenged with the RCASBP(A)-EGFP virus and virus infection was monitored as described in the legend to Fig. 2.

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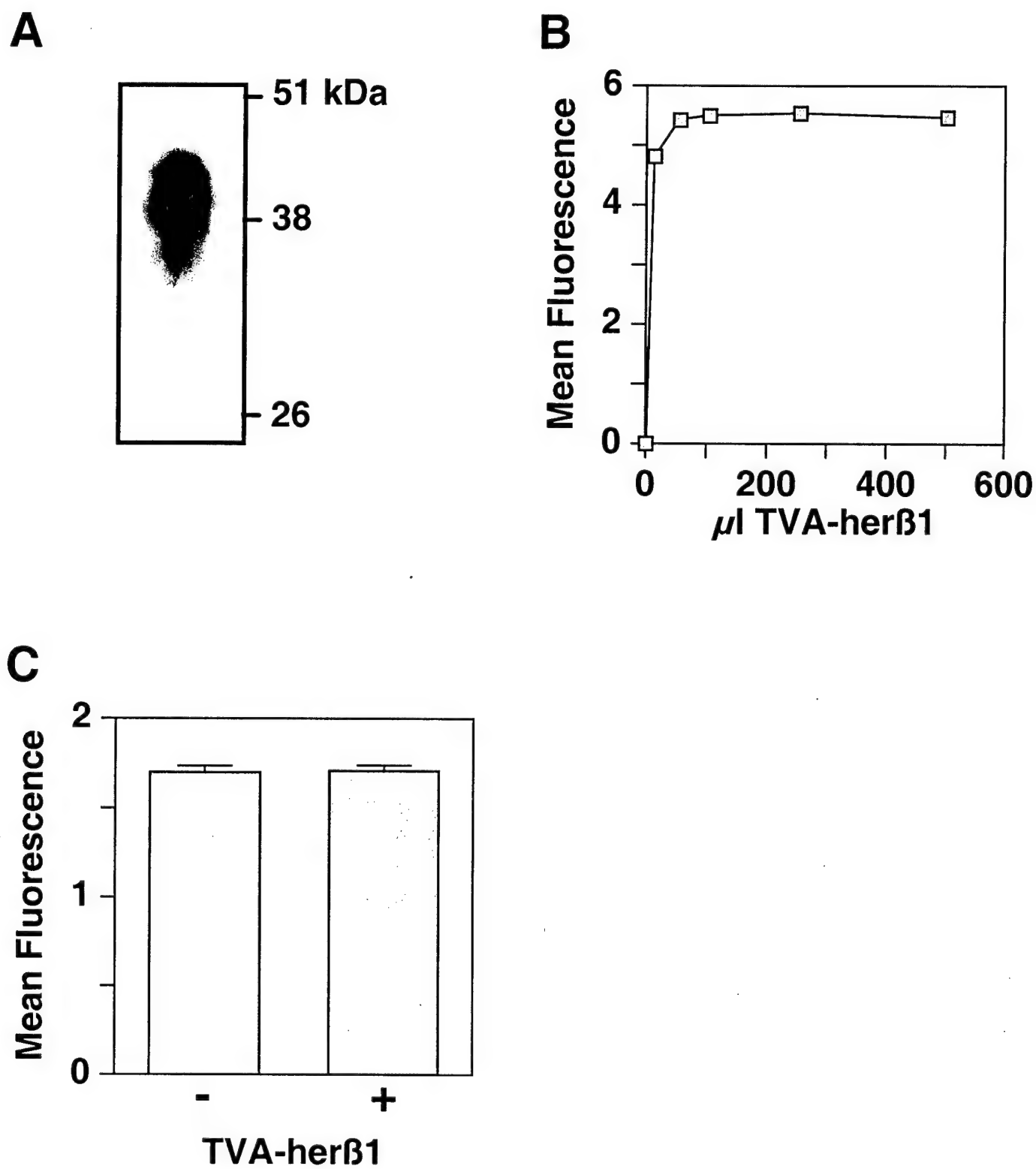


Figure 1

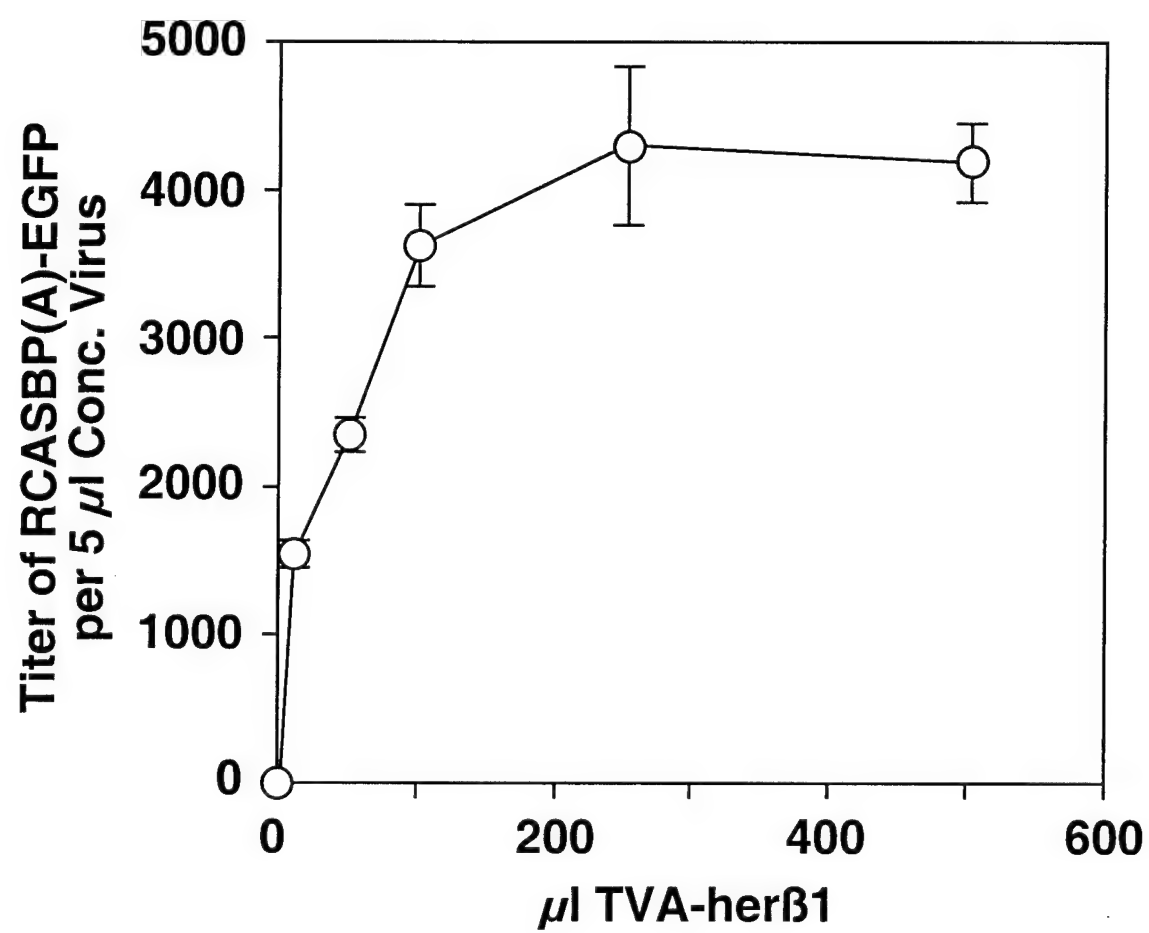
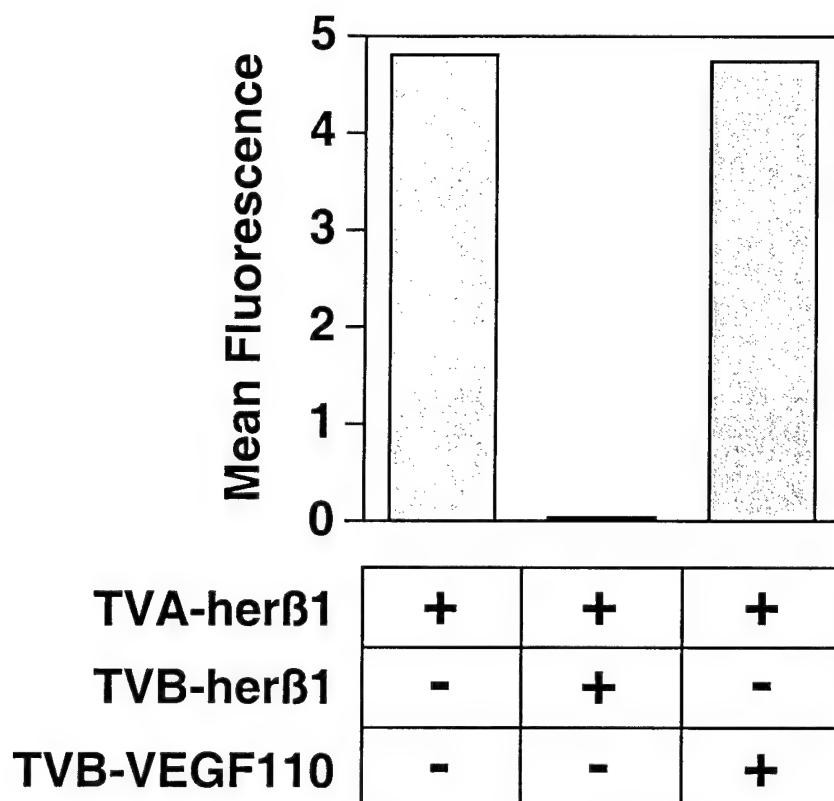
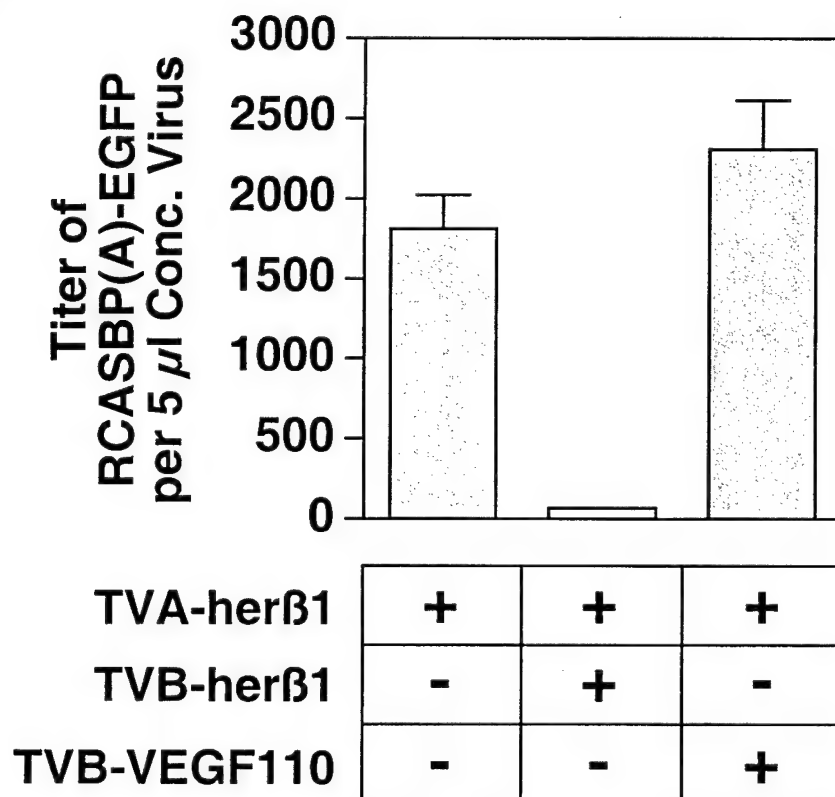


Figure 2

**A****B****Figure 3**

Cell Type	Efficiency of ASLV-A Infection (%)	
	-TVA-her $\beta$ 1	+TVA-her $\beta$ 1
NIH 3T3	0.07	0.05
N234	0.11	5.04
NIH 3T3-TVA <sup>syn</sup>	100	N.D.

**Table 1. TVA-her $\beta$ 1 mediates specific and efficient viral entry into N234 cells.** NIH 3T3 cells and N234 cells were incubated with or without TVA-her $\beta$ 1 before challenge with the subgroup A ASLV vector RCASBP(A)-EGFP encoding the enhanced green fluorescent protein. The relative numbers of EGFP-transduced cells were determined by flow cytometric analysis and are shown as compared with that number obtained when the same amount of virus was used to infect NIH 3T3-TVA<sup>syn</sup> cells ( $9.6 \times 10^6$  EGFP transducing units/ml (100-fold) concentrated virus, defined here as 100% infection).

# Appendix 5

## Targeting Retrovirus Vectors Using Molecular Bridges

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## INTRODUCTION

Approaches have been developed to target retrovirus vectors to specific cell types that do not involve modifying the viral envelope (Env) protein but instead employ molecular bridges to couple wild-type Env to specific cell surface receptors. To date, several types of molecular bridge have been described. The first is formed by crosslinked antibodies and ligands that link Env to a specific cell surface receptor. The second type is a chimeric bridge protein comprised of the functional domain of a retroviral receptor fused in-frame to either a ligand or a single chain antibody. In this chapter, I will review the progress made using each of these methods and will provide a perspective upon the future use of this viral-targeting approach.

## RETROVIRAL ENTRY

Retroviral entry is mediated by Env proteins which consist of surface (SU) subunits involved in receptor-binding and transmembrane (TM) subunits involved in the fusion of virus and cell membranes. These proteins exist as a metastable trimer of heterodimers on the viral surface and for most retroviruses they are thought to undergo fusogenic

conformational changes following specific interactions with cell surface receptors and/or coreceptors (Sommerfelt, 1999).

Retroviruses have evolved to use a variety of different cell surface proteins as their receptors. These include type-1 transmembrane proteins of the immunoglobulin-, low density lipoprotein receptor-, and tumor necrosis factor receptor-protein families (Sommerfelt, 1999). Also, several multiple membrane spanning proteins that function as chemokine receptors or as transporters of amino acids or ions have been exploited as retroviral receptors and coreceptors (Sommerfelt, 1999).

Studies of the human immunodeficiency virus (HIV-1) and avian sarcoma and leukosis viruses (ASLVs) have revealed that the receptor-interaction is not only important for virus binding but it also leads to structural and functional changes in Env necessary for the subsequent fusion of viral and cellular membranes. In the case of HIV-1, viral entry seems to occur by direct fusion of the viral membrane with the host cell plasma membrane following the sequential interaction of Env with the CD4 receptor and then with CC or CXC chemokine receptors (coreceptors) (Wyatt, et al., 1998). CD4-binding induces structural changes in HIV-1 SU that lead to exposure of the chemokine receptor-binding site on the viral

glycoprotein, thus promoting downstream steps of viral entry (Wyatt, et al., 1998).

By contrast to HIV-1, ASLVs enter cells by a low pH-dependent mechanism that most likely involves virus trafficking to an acidic endosomal compartment where fusion occurs (Mothes, et al., 2000). The TVA receptor for subgroup A-ASLV (ASLV-A) triggers structural changes in the viral glycoprotein that alter the conformation of SU (Gilbert, et al., 1995; Damico, et al., 1999), lead to the high affinity binding of the viral glycoprotein to membranes (Hernandez, et al., 1997; Damico and Bates, 1998), and prime the viral glycoprotein for low pH fusion activation (Mothes, et al., 2000).

These two examples of retrovirus-receptor interactions clearly illustrate the fact that retroviral receptors are not only involved in virus-binding but they also initiate changes in the viral glycoprotein that are necessary for membrane fusion. This post-binding function of retroviral receptors may explain why it has been so difficult to target retroviral vectors to novel cell surface receptors using recombinant ligand-Env fusion proteins (Zhao, et al., 1999) (see also chapters 14 and 15). In this case, ligands also have to be accommodated in sites of Env where they

do not interfere with protein biosynthesis and transport to cell membrane for virion incorporation. In designing a retrovirus targeting approach it may therefore be desirable to leave the envelope glycoprotein untouched while taking advantage of molecular bridges that can couple virions to the target cell membrane. To date, several different types of molecular bridge have been described, namely cross-linked antibodies and ligands, as well as retroviral receptor-ligand bridge proteins.

## MOLECULAR BRIDGES

### Cross-Linked Antibodies and Ligands

The first types of molecular bridge that were employed for viral targeting were cross-linked antibodies that attached Env to specific cell surface proteins. This approach relied upon binding an Env-specific antibody to virion surfaces and binding another antibody or a ligand to a specific cell surface marker (Figure 1). These bound reagents were then crosslinked by another antibody (Goud, et al., 1988) or by streptavidin (Roux, et al., 1989; Etienne-Julan, et al., 1992) (Figure 1). This approach has been tested with ecotropic murine leukemia virus (MLV-E) vectors and with antibodies and ligands that bind to a variety of different cell surface

markers including the transferrin receptor, major histocompatibility complex (MHC) class I and class II proteins, the epidermal growth factor receptor (EGFR) and the insulin receptor (Goud, et al., 1988; Roux, et al., 1989; Etienne-Julan, et al., 1992) (Table 1).

Taken together, these studies revealed that retroviral targeting can be achieved through the use of Env-specific antibody-containing molecular bridges (Roux, et al., 1989; Etienne-Julan, et al., 1992). However, this method usually led to no viral infection or only to an extremely low level of targeted viral infection (Table 1).

A modification to this approach has involved using a fusion protein comprised of a cell type-specific ligand fused to protein A (Etienne-Julan, et al., 1992). This type of fusion protein should be capable of binding directly to a cell surface ligand-receptor and to the Fc portion of an Env-specific thus eliminating the need for an additional crosslinking agent. However, this approach has so far failed to give rise to targeted viral entry (Etienne-Julan, et al., 1992).

In summary, molecular bridges that contain Env-specific antibodies have, at least in some circumstances, been shown to lead to targeted

retroviral infection. However, this approach has worked only at a low efficiency. Presumably this is because the Env-specific antibodies fail to induce the post-binding changes in the viral glycoprotein which occur upon contact with the natural viral receptor and that are required for membrane fusion.

### Retroviral Receptor-Ligand Bridge Proteins

A different type of molecular bridge that has been described is a retroviral receptor-ligand bridge protein. These proteins consist of the extracellular domain of a retroviral receptor fused to a ligand or a single-chain antibody. These bridge proteins are bifunctional reagents that attach to specific cell surface receptors through their ligand moiety and to the viral glycoprotein through the retroviral receptor domain (Figure 2). Importantly, these bridge proteins preserve the contact between the natural viral receptor and Env, thus increasing the likelihood that efficient targeted viral infection can be achieved.

To date, two distinct types of bridge protein have been described, based upon either the mCAT-1 receptor for MLV-E, or instead upon the TVA and TVB receptors for ASLVs.

## Ecotropic MLV Receptor

The cellular receptor for MLV-E is mCAT-1, a cationic amino acid transporter with multiple membrane-spanning domains (reviewed in Sommerfelt, 1999). Determinants that are important for viral entry were mapped in mCAT-1 by exchanging amino acid residues between the murine receptor and its human homolog which is not normally a viral receptor. These studies showed that it was possible to convert the human protein into a functional MLV-E receptor by replacing several amino acid residues within the predicted third extracellular domain (Albritton, et al., 1993; Yoshimoto, et al., 1993).

Meruelo and colleagues have used this modified form of the human protein in an attempt to generate a bridge protein for retroviral targeting. Specifically they constructed TGF $\alpha$ -mH13, a soluble chimeric protein containing the first 50 amino acids of transforming growth factor- $\alpha$  (TGF $\alpha$ ) fused-in-frame to the third extracellular domain of the modified form of the human homolog of mCAT-1 (Ohno, et al., 1995). They showed that TGF $\alpha$ -mH13, immobilized on a 96-well plate or attached to cell surface EGFR, was capable of binding to MLV-E (Ohno, et al., 1995). However, they have

not shown that this bridge protein can target viral infection via the EGFR (Ohno, et al., 1995), probably because the third extracellular domain of the modified human protein cannot support viral entry when it is removed from its context within the multiple membrane-spanning receptor.

### ASLV Receptors

ASLVs are divided into at least ten different viral subgroups based upon receptor-usage, host-range, and sensitivity to neutralizing antibodies. To date, cellular receptors for four of these viral subgroups (ASLV-A, -B, -D, and -E) have been identified. TVA, the cellular receptor for ASLV-A, contains a single LDL-A module and is a member of the low density lipoprotein receptor family (Bates, et al., 1993; Bates, et al., 1998). TVB, the cellular receptor for subgroups B, D, and E ASLV, is a death receptor of the tumor necrosis factor receptor superfamily that is most closely related to the mammalian TRAIL receptors, DR4 and DR5 (Brojatsch, et al., 1996; Smith, et al., 1998; Adkins, et al., 1997; Adkins, et al., 2000; Schulze-Osthoff, et al., 1998).

ASLV receptors are especially well suited for a viral targeting strategy that employs retroviral receptor-ligand bridge proteins because

they can render a variety of different mammalian cell types susceptible to viral infection. Furthermore, in contrast to most other retrovirus receptors which, like mCAT-1, have multiple membrane-spanning domains, ASLV receptors are simple type-1 transmembrane proteins and therefore all of the functional determinants needed for virus interaction are contained within their single extracellular domains (Sommerfelt, 1999). Therefore, the extracellular domains of these receptors can be produced as soluble forms that retain the ability to bind virus and stimulate entry. As discussed below, ASLV receptor-ligand bridge proteins containing EGF, vascular endothelial growth factor (VEGF), heregulin $\beta$ 1, or a single chain antibody moiety (MR1), can support efficient and specific virus entry into cells that express the cognate target receptors.

## EGF

EGF was chosen as the prototypical ligand to test the feasibility of targeting retroviral infection via ASLV receptor-ligand bridge proteins (Snitkovsky and Young, 1998; Boerger, et al., 1999). This was because EGF is a small ligand only 53 amino acids long and there was a concern that large bulky ligands might sterically interfere with virus entry (e.g., Buchholz, et al., 1996). Also, the interaction between this ligand and its

cellular receptor, EGFR-a tyrosine kinase receptor, was well characterized and several different receptor mutants were available that exhibited different cellular trafficking properties upon binding ligand (DiFiore and Gill, 1999). More importantly the EGFR, and related proteins that serve as heregulin receptors (erbB2, erbB3, and erbB4), are often overexpressed in human cancers (Yarden and Sliwkowski, 2001), making this type of receptor a clinically relevant target for a retroviral gene delivery approach.

Two synthetic genes were constructed encoding bridge proteins with the extracellular domains of either TVA (TVA-EGF) or TVB (TVB-EGF) fused in-frame to a proline-rich hinge region and to EGF (Snitkovsky and Young, 1998; Boerger, et al., 1999). These chimeric proteins were shown to be capable of bridging ASLV SU to either wild-type, or kinase-deficient, forms of the EGFR expressed on the surfaces of transfected mouse L cells (Snitkovsky and Young, 1998; Boerger, et al., 1999). This binding was competed by EGF, confirming that the bridge protein was attached to the ligand-binding region of the EGFR (Snitkovsky and Young, 1998; Boerger, et al., 1999). Also like EGF, TVA-EGF was rapidly internalized into cells via the wild-type, but not the kinase-deficient EGFR, demonstrating that this bridge protein behaves in much the same way as the normal physiological ligand (Snitkovsky and Young, 1998).

The TVA-EGF and TVB-EGF bridge proteins were also shown to promote specific infection of cells when attached to cell surface EGFR prior to viral challenge. The level of targeted viral infection ranged from approximately 6% to 54% of that level obtained with control cells expressing transmembrane forms of these receptors (Snitkovsky and Young, 1998) (Table 2). Intriguingly, the level of TVA-EGF-dependent viral infection mediated by the kinase-deficient EGFR was approximately 2.5-fold higher than that level seen via the wild-type EGFR (Table 2) (Snitkovsky and Young, 1998). While the reason for this difference between both forms of the receptor is not yet known, it may be related to the fact that these receptors are trafficked differently upon binding ligand. The ligand-bound form of the wild-type EGFR is rapidly mobilized out of cell surface caveolae, which are membrane microdomains rich in cholesterol and sphingolipids, and becomes incorporated into clathrin-coated pits where it is rapidly endocytosed. By contrast, the ligand-bound kinase-deficient receptor remains associated with caveolae for a longer time period and it is internalized more slowly (DiFiore, 1999).

The results obtained with TVA-EGF and TVB-EGF were encouraging since they demonstrated that efficient and specific viral

targeting can be achieved by adding ASLV receptor-ligand bridge proteins to cells before viral challenge. In the case of TVB-EGF, it has also been possible to achieve targeted viral infection by preloading pseudotyped MLV particles containing ASLV-B Env with TVB-EGF. These preloaded virions displayed a remarkable specificity for cells that expressed the EGFR (Boerger, et al., 1999) (Table 2). Indeed, the level of targeted virus infection that was achieved with cells expressing the kinase-deficient EGFR was greater than that seen with control cells expressing a transmembrane form of TVB (Boerger, et al., 1999) (Table 2). Also, as before, the level of infection that was observed with cells expressing the kinase-deficient EGFR exceeded that seen with cells expressing the wild-type protein (Boerger, et al., 1999) (Table 2). It was also shown that TVB-EGF-loaded virions can be produced directly from viral vector packaging cells (that express the bridge protein along with all of the components of a virus vector) and that these virions retain their targeting specificity for cells that express the EGFR (Boerger, et al., 1999).

### Heregulin $\beta$ 1

Based upon the results obtained with ASLV receptor-EGF bridge proteins, it was reasoned that replacing the EGF ligand with heregulin  $\beta$ 1

should lead to targeted viral infection toward cells that express EGFR-related heregulin receptors (erbB2/erbB3/erbB4). Heregulin receptors are formed by erbB3 or erbB4 homodimers of or instead by erbB2/erbB3 and erbB2/erbB4 heterodimers (Stern, 2000).

This idea was tested by fusing the EGF-like domain of heregulin  $\beta 1$  to the extracellular domain of TVA generating the TVA-her $\beta 1$  fusion protein (Snitkovsky and Young, manuscript submitted). This fusion protein bound specifically to transfected NIH 3T3 cells that express erbB2 and erbB3 and rendered these cells susceptible to infection by ASLV-A vectors (Snitkovsky and Young, manuscript submitted) (Table 2). Therefore, an ASLV receptor-heregulin  $\beta 1$  fusion protein can support targeted viral entry into cells that express heregulin receptors.

## VEGF

VEGF was chosen as the next type of ligand to test because its receptors are expressed predominantly on endothelial cells. Therefore, any viral targeting strategy that employs VEGF has the potential for delivering genes specifically to endothelial cells and this may have clinical relevance e.g. in allowing for gene delivery into cells of the tumor

vasculature. Several different VEGF receptors with cytoplasmic tyrosine kinase domains have been described: VEGFR-1 (flt-1), VEGFR-2 (KDR/flk-1), and neuropilins-1, and -2 (Neufeld, 1999). The non-tyrosine kinase receptor VEGFR-3 (flt-4) also serves as a VEGF receptor (Neufeld, et al., 1999).

VEGF was also of interest to test since it is a much more complex ligand than EGF. VEGF is a member of the cysteine-knot growth factor superfamily and it is expressed as an antiparallel disulfide-linked homodimer with two receptor-binding sites located at opposite ends of the molecule (Neufeld, et al., 1999). Alternative splicing of a primary mRNA transcript gives rise to five distinct VEGF isoforms designated as VEGF<sub>121</sub>, VEGF<sub>145</sub>, VEGF<sub>165</sub>, VEGF<sub>189</sub>, and VEGF<sub>206</sub> (Neufeld, et al., 1999). Each of these VEGF isoforms (with the exception of VEGF<sub>121</sub>) binds extracellular membrane-associated heparan sulfate-containing proteoglycans (Neufeld, et al., 1999). Since this feature would be expected to reduce the target cell specificity of infection via a VEGF-containing bridge protein, a modified form of this ligand (designated as VEGF<sub>110</sub>) lacking the C-terminal heparin binding domain was used to construct the TVA-VEGF<sub>110</sub> bridge protein (Snitkovsky, et al., 2000).

TVA-VEGF110 was shown to bind to ASLV-A SU and to porcine aortic endothelial cells that had been transduced with a retroviral vector encoding VEGFR-2 (Snitkovsky, et al., 2001). TVA-VEGF110 also supported efficient viral infection when it was added to these cells prior to viral challenge (Table 2). The level of targeted viral infection achieved was approximately 9% of that level seen with control cells that expressed a transmembrane form of the TVA receptor (Table 2) (Snitkovsky, et al., 2001). Therefore, it is possible to efficiently target retroviral infection via VEGF receptors, using an ASLV receptor-VEGF bridge protein.

#### An ASLV Receptor-Single Chain Antibody Bridge Protein

While targeted retroviral infection via ASLV receptor-ligand bridge proteins is useful, there are several potential limitations of this approach. For example, this approach limits the sites of virus attachment to the ligand-binding regions of cellular receptors and this may allow viral targeting in some, but not other, instances. Furthermore, by definition the use of ligand-containing bridge proteins is limited to those receptors with known ligands. It was therefore of interest to test whether targeted retroviral infection could also be achieved using a bridge protein that contains a single chain antibody moiety in place of the ligand. Single chain

antibodies have the advantage that they can be directed against a variety of sites on a target cellular receptor as well as against cellular factors for which there are no known ligands.

The single chain antibody-containing bridge protein that has been tested was TVA-MR1 which consists of the extracellular domain of TVA fused to the MR1 single chain antibody (Snitkovsky, et al., 2000). MR1 binds specifically to EGFRvIII, a mutant form of the EGFR that is expressed on a variety of different human cancer cell types including those from glioblastoma, ovarian cancer, prostate cancer, lung cancer and breast cancer (Huang, et al., 1997). EGFRvIII results from a common deletion/rearrangement that occurs during amplification of the EGFR gene during tumor biogenesis. As a result, the protein exhibits an in-frame deletion of 267 amino acids from the extracellular domain and it is constitutively active in the absence of ligand-binding (Huang, et al., 1997). The MR1 antibody was raised against a synthetic peptide sequence that spans the two segments of the EGFR that are brought together as a consequence of this genetic rearrangement (Lorimer, et al., 1996).

TVA-MR1 was shown to bind to ASLV-A SU and to human 293 cells that had been engineered to express a murine form of EGFRvIII

(Snitkovsky, et al., 2000). This binding was specifically blocked by a synthetic peptide containing the MR1 epitope but was unaffected by a scrambled version of this peptide (Snitkovsky, et al., 2000). Furthermore, the bridge protein did not bind to the parental human 293 cells confirming that it binds specifically to EGFRvIII (Snitkovsky, et al., 2000).

EGFRvIII-expressing cells preloaded with TVA-MR1 were very susceptible to infection by ASLV-A vectors (Snitkovsky, et al., 2000). The level of targeted viral infection that was achieved was approximately 8-9% of that level obtained with cells that express a transmembrane form of TVA (Snitkovsky, et al., 2000) (Table 2). TVA-MR1-dependent infection was specifically blocked when the bridge protein was incubated with the antibody epitope-containing peptide before it was added to cells, confirming that infection required the MR1-EGFRvIII contact (Snitkovsky, et al., 2000).

## CONCLUSIONS AND POTENTIAL CLINICAL APPLICATION

The studies described in this chapter have shown that retroviruses can be targeted to specific cell types by using molecular bridges. In those instances where an Env-specific antibody has been employed as a

bridging component, targeted viral infection has been achieved but in general the levels of infection obtained are too low to be generally useful. By contrast, consistently high levels of targeted infection have been achieved through the use of ASLV receptor-ligand bridge proteins. Those bridge proteins containing the ligands EGF, VEGF, heregulin  $\beta 1$ , or an EGFRvIII-specific single chain antibody all functioned well to allow targeted viral infection when they were added to cells before viral challenge. In addition, in the case of TVB-EGF, it has been shown that targeted viral infection can be achieved with bridge protein-loaded virions. It is important to note that this method is not limited to the use of just ASLV vectors because ASLV Env proteins can be incorporated onto other retroviral vectors including those based upon MLV to create viral pseudotypes (e.g., see Boerger, et al., 1999). It should also be possible to create viral pseudotypes with a lentiviral core and with ASLV Env proteins in order to target non-dividing cell types for infection.

ASLV receptor-ligand bridge proteins have provided a versatile system for targeting retrovirus infection to specific cell types. We have recently designated these proteins as "GATEs" (Guided Adaptors for Targeted Entry) (Snitkovsky and Young, in preparation). By changing the nature of the ligand, these reagents should be useful for targeting

retroviral vectors to a variety of different cell types. By exchanging the viral receptor domain of this type of bridge protein, it should also be possible to target a number of other viral vectors to specific cell types. Indeed, other researchers have recently described a similar approach that has employed soluble coxsackie B and adenovirus receptor (CAR)-EGF/Fc fusion proteins to target adenovirus vectors to cells that express cognate receptors (Dmitriev, et al., 2000; Ebbinghaus, et al., 2001).

The results to date with soluble ALV receptor-ligand bridge proteins have all been obtained *in vitro* using bridge protein-loaded virions or instead using cultured cells that have been preloaded with the bridge protein prior to viral challenge. While these results are encouraging there are still a number of significant hurdles that must be overcome before this can be considered a useful method for targeting retrovirus vectors in *in vivo* gene therapy protocols. As with other retroviral vector targeting protocols one of the most difficult challenges will be to generate viral vector stocks with sufficiently high titer and excellent bioavailability. The high titer problem may be overcome by subjecting virions to a selection protocol in order to identify those with altered Env proteins that can more efficiently mediate bridge protein-dependent viral entry. Nevertheless, the results obtained with ALV receptor-ligand bridge proteins represent an

important first step toward the application of molecular bridges for cell-specific gene therapy using retroviral vectors.

## ACKNOWLEDGEMENTS

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## FIGURE LEGENDS

Figure 1. Retroviral targeting via Env-specific antibodies crosslinked to an antibody or to a ligand that binds to a specific cell surface receptor. The protocol shown here was taken from Goud, et al., 1988; Roux, et al., 1989; and Etienne-Julan, et al., 1992.

Figure 2. Retroviral targeting via retroviral receptor-ligand bridge proteins. The bridge protein consists of the functional domain of a retroviral receptor fused via a proline-rich hinge region to a ligand or to a single chain antibody (Snitkovsky & Young, 1998; Boerger, et al., 1999; Snitkovsky, et al., 2000; and Snitkovsky, et al., 2001).

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## TABLE LEGENDS

Table 1. The efficiency of retroviral targeting mediated by cross-linked antibodies and ligands. The numbers shown from Roux 1989 and Etienne-Julan 1990 were obtained by comparing virus infection efficiency to that obtained with NIH-3T3 cells.

Table 2. Efficiency of viral targeting mediated by ASLV receptor-containing bridge proteins. The data used to generate this table was derived from the publications that are indicated. The efficiency of infection shown is compared relative to that seen with the same amount of virus added to matched cells that were engineered to express transmembrane forms of the TVA and the TVB receptors (100% infection control).

TABLE 1.

Virus-Specific Antibody	Crosslinking Agent	Cellular Marker-Specific Antibody/Ligand	Cell Type	Efficiency (%)	Reference
anti-MLV SU	secondary antibody	anti-human transferrin receptor	Hep 2	0	Goud et al. 1988
biotinylated anti-ecotropic MLV SU	streptavidin	biotinylated anti-MHC class I	HeLa	0.01-0.16	Roux et al. 1989
		biotinylated anti-MHC class II	Hela	0.2-0.7	
		biotinylated anti-EGFR	A431	1.4-2.4	Etienne-Julan et al. 1990
		biotinylated anti-transferrin receptor	HepG2	<0.002	
		biotinylated-EGF	A431	0.08-0.23	
		biotinylated-insulin	HeLa	0.016	
		biotinylated-asialofetuin	HepG2	<0.002	
none	multivalent lectins	none	Hela	<0.002	

TABLE 2.

ASLV receptor- bridge protein	Preloaded on to	Target receptor	Efficiency of infection (%)		References
			- Bridge protein	+ Bridge protein	
TVA-EGF	Cells	control L cells (EGFR- minus)	0	0	Snitkovsky and Young 1998
		L cells: WT-EGFR	0	6.60	
		L Cells: ATP binding mutant-EGFR	0	16.5	
TVB-EGF	Virions	control L cells: (EGFR- minus)	0	54.61	Boerger et al. 1999
		L cells: WT-EGFR	0.04	0.03	
		L cells: ATP binding mutant-EGFR	0.01	28.12	
TVA-her $\beta$ 1	Cells	control NIH 3T3 cells (erbB2/3-minus)	0.06	137.50	Snitkovsky and Young (manuscript submitted)
		NIH 3T3 cells: erbB2/erbB3	0.07	0.05	
		control PAE cells (VEGFR-minus)	0.1	5.04	
TVA-VEGF	Cells	control PAE cells (VEGFR-minus)	0.01	0.09	Snitkovsky et al. 2001
		PAE cells: VEGFR-2	0.02	8.72	
		control 293 cells (EGFRvIII-minus)	0.05	0.04	
TVA-MR1		293 cells: EGFRvIII	0.05	8.38	Snitkovsky et al. 2000

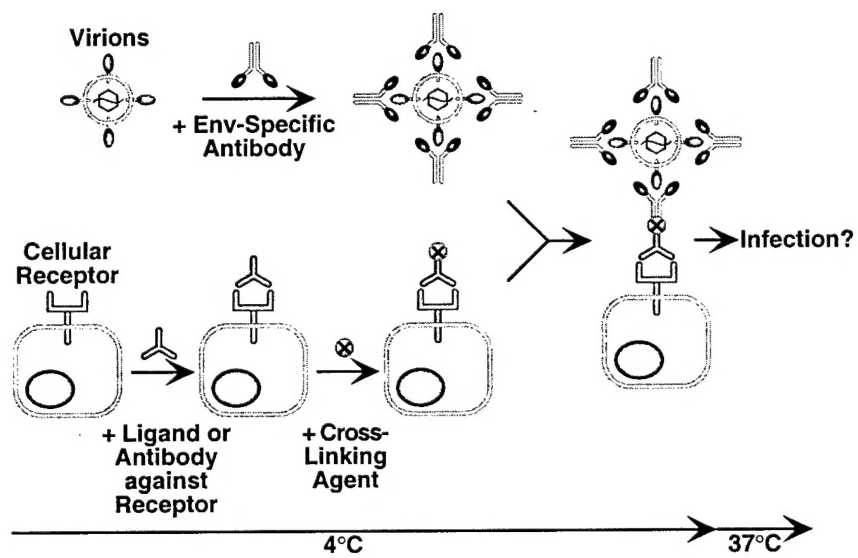


Figure 1

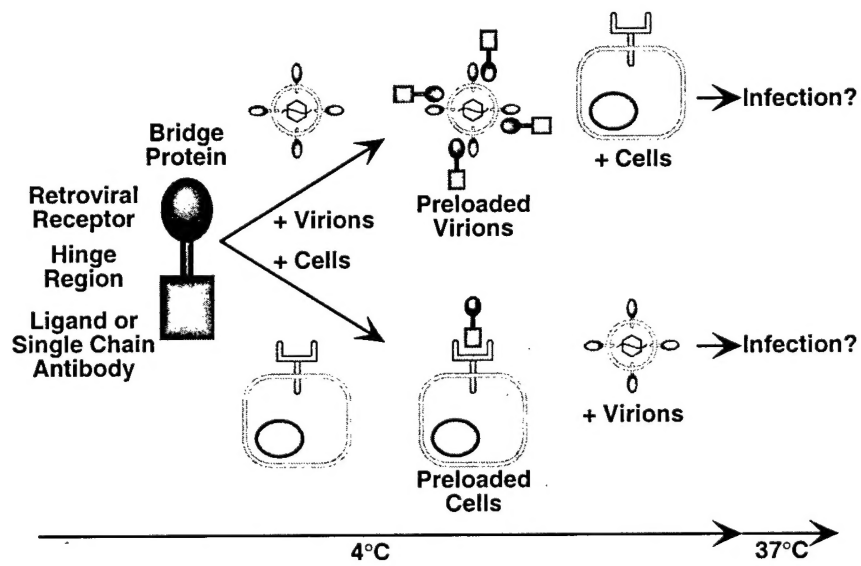


Figure 2